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THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY

WOODLAND AVENUE AND THIRTY-SIXTH STREET, PHILADELPHIA 4, PA.

THE RENAL EXCRETION OF SODIUM, POTASSIUM, AND WATER BY THE HARBOR SEAL (PHOCA VITULINA L.): EFFECT OF APNEA; SODIUM, POTASSIUM, AND WATER LOADING; PITRESSIN; AND MERCURIAL DIURESIS[1]

S. E. BRADLEY, G. H. MUDGE AND W. D. BLAKE[2]
The Mt. Desert Island Biological Laboratory, Salisbury Cove, Maine; the Department of Medicine, College of Physicians and Surgeons, Columbia University; and the Presbyterian Hospital, New York, New York

SIX FIGURES

Aquatic mammals living in the sea have been obliged to adapt to a peculiarly difficult way of life. The search for food demands long periods of submersion and a remarkable resistance to asphyxia. Irving and his coworkers (Irving, '38, '39; Irving et al., '42) have shown that various circulatory adjustments occur in diving mammals that reduce the vascular system to a heart-lung-brain circulation, effectively conserving oxygen held in the blood and pulmonary air spaces for the use of tissues sensitive to lack of oxygen. This response has been found to entail marked vasoconstriction in the kidney with almost complete cessation of glomerular filtration and urine formation (Bradley and Bing, '42). Renal vasomotor activity is likewise concerned in the conservation of water and electrolytes. Hiatt and Hiatt ('42) observed a striking increase in

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[1] Aided by the Edward N. Gibbs Prize Fund, New York Academy of Medicine, New York, New York. A preliminary report of this work was made at the annual meeting of the American Physiological Society, 1950 (Bradley et al., '50).

[2] Post-doctorate Research Fellow of the U. S. Public Health Service; present address: Department of Physiology, University of Oregon, School of Medicine, Portland, Oregon.

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the renal blood flow and glomerular filtration rate in young harbor seals following a meal of herring. These animals (and presumably other marine animals) do not drink sea-water, but depend entirely for water upon the metabolic water and the body fluids of ingested fish (Irving et al., '35). The Hiatts ('42) suggest that the post-prandial renal vasodilation may permit excretion of wastes and excess electrolytes only when water is available for urine formation, vasoconstriction and the associated reduction in glomerular filtration limiting water excretion at other times. The results reported here of a study of renal excretion of varying loads of water and electrolyte at different filtration rates in seals, support this view. It was found that sodium, potassium and water excretion decreased greatly (often indeed to a disproportionate degree) with reduction in filtration during apnea, regardless of load. Special physiologic mechanisms for preferential elimination of sodium and potassium were not demonstrable under the condition of these experiments.

METHODS

All studies were made upon young unanesthetized female seals [3] weighing between 30 and 40 pounds. The animals were held securely by straps in the dorsorecumbent position throughout each experiment. The glomerular filtration rate was measured by the creatinine clearance (Smith, '36; Bradley and Bing, '42). An indwelling catheter was placed in the bladder and creatinine (5 gm) was given by stomach tube. After a period of 30 to 45 minutes to permit establishment of appropriate plasma creatinine concentrations, the bladder was emptied and urine collected thereafter at regular intervals and carefully measured. No attempt was made to wash out the bladder in these experiments, but air was injected into the bladder at each collection in order to assure complete emptying. Blood samples were taken from a flipper vein at the beginning and at intervals of approximately 30 minutes throughout the experiment. Every effort was made to avoid hemolysis. However, there is no reason to believe that hemolysis had any significant effect upon the determination of plasma electrolytes since the sodium and potassium concentrations in whole blood and plasma were approximately equal. Creatinine concentrations were determined in cadmium filtrates (Fujita and Iwatake, '31) of plasma and in aliquots of diluted urine by the Bonsnes-Taussky method ('45) and clearance values calculated in the customary manner. A flame photometer, using an internal lithium standard, was used to measure the sodium and potassium content of diluted plasma and urine samples.

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[3] We are indebted to Mr. Gordon Davis and Mr. P. Goggins, of the U. S. Fish and Wildlife Service, and Mr. H. Smith, Superintendent of Fisheries, Boothbay Harbor, Maine, for supplying the animals used in this study.

Herring, cod-liver oil or condensed milk were administered by force-feeding or by stomach tube to assure high filtration rates in 13 of 24 studies on 6 seals. In order to study the effect of low filtration rates on water and electrolyte excretion asphyxia was induced at some time in 18 experiments by halting respiration with a closed cone held over the muzzle. Apnea was maintained intermittently for 10 to 15 minutes or longer, allowing the animal to take two or three breaths every two or three minutes. A water diuresis was induced in 6 animals by from 500 to 1500 cm^3 of water in divided doses administered by stomach tube. Pitressin (a total dose of 800 to 1000 milliunits) was given subcutaneously and intravenously in divided doses on 4 occasions. In 4 experiments sodium chloride (10 to 20 gm) dissolved in about 300 cm^3 of water was given by stomach tube in order to raise the serum sodium concentration and to impose a load of sodium ion on the kidney for excretion. Potassium chloride (15 gm) was given daily to two seals for 3 and 5 days, respectively. On the day of study, 10 and 20 gm doses were given by stomach tube 45 minutes before making measurements. One of these animals died shortly after clearance measurements were started as a result of potassium intoxication (plasma potassium concentration of 17 mEq/L). A third seal received 20 gm of potassium chloride just prior to study. In 5 experiments, a mercurial diuretic (Salyrgan 1 cm^3) was administered intravenously just before or shortly after beginning clearance measurements. None of the animals ate well and all lost weight during the month of study each summer ('48, '49). However, they appeared to be in vigorous healthy condition at the time of each experiment.

RESULTS

The studies reported below were designed primarily to test the effect of the large changes in filtration, physiologically possible in the seal, on the urinary excretion of water, sodium and potassium. Since a so-called basal state is difficult to define and perhaps impossible to obtain in these animals, it seemed unlikely that comparison of the individual values obtained in the study of the small number of animals at our disposal would clarify the importance of filtration in water and electrolyte excretion. Rather, it appeared that imposition of filtration changes upon some maximal activity of the tubules would be more illuminating. A number of factors play important roles in altering tubular activity and in determining excretory rate independent of filtration rate (Smith, '51). Among these may be numbered the state of hydration, electrolyte loading, endocrine activity, hemodynamic status and plasma concentrations, and it is probable that there are others, still unrecognized and uncharacterized. The operation of these factors may mask completely the influence of filtration change on excretion when changes are relatively small. No attempt has been made in this study to evaluate the effect of such small changes. However, it has been possible to demonstrate the overwhelming influence of large changes in filtration independently of tubular activity.

The elevated filtration rate following feeding was employed as a baseline against which the reduction during apnea could be matched. Since feeding involved a relatively small, but variable and uncontrolled intake of sodium, potassium and water, no effort was made to test the effect of post-prandial hyperfiltration alone. The renal responses to a mercurial diuretic agent, anti-diuretic hormone and large loads of water, sodium or potassium were studied individually in order to assess the importance of the tubular component in urinary excretion relative to filtration. These responses will be considered first and the effect of the filtration change induced by apnea will then be discussed.

Mercurial diuresis. Salyrgan, a mercurial diuretic, was administered in doses of 1.0 cm³ subcutaneously and intraven-

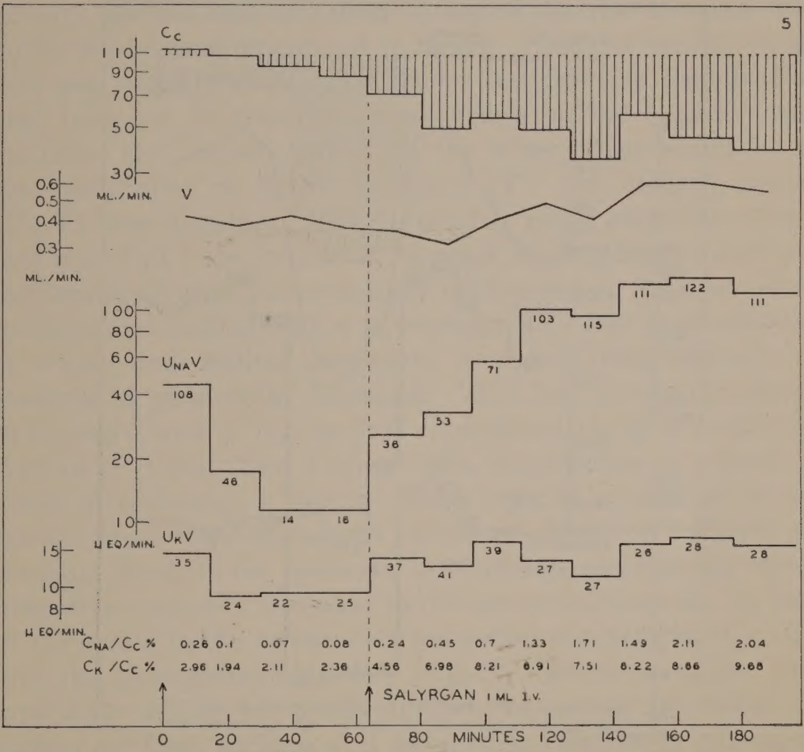

Figure 1 The effect of a mercurial diuretic (Salyrgan) on glomerular filtration, urine flow and electrolyte excretion in the harbor seal.

Figures for glomerular filtration rate (creatinine clearance — C_c), urine flow (V), sodium excretion ($U_{Na}V$), and potassium excretion (U_KV) are plotted semilogarithmically against time. Values for the urinary concentrations of sodium and potassium during each collection period are inserted here below the graphs of sodium and potassium outputs, respectively. At the bottom of the illustration the sodium and potassium-creatinine clearance ratios (per cent of filtered electrolyte excreted in the urine) are presented.

Following the administration of Salyrgan the sodium and water output rose. Potassium excretion increased somewhat and levelled off. Filtration decreased probably without relation to the effect of the drug since it was falling during the control periods from a high value presumably the result of feeding prior to the experiment.

ously in divided doses in 5 experiments. The effect of the drug was observed during the response to sodium, potassium and water loading, on one occasion each. Apnea was induced during these three and during one additional study following injection of the diuretic.

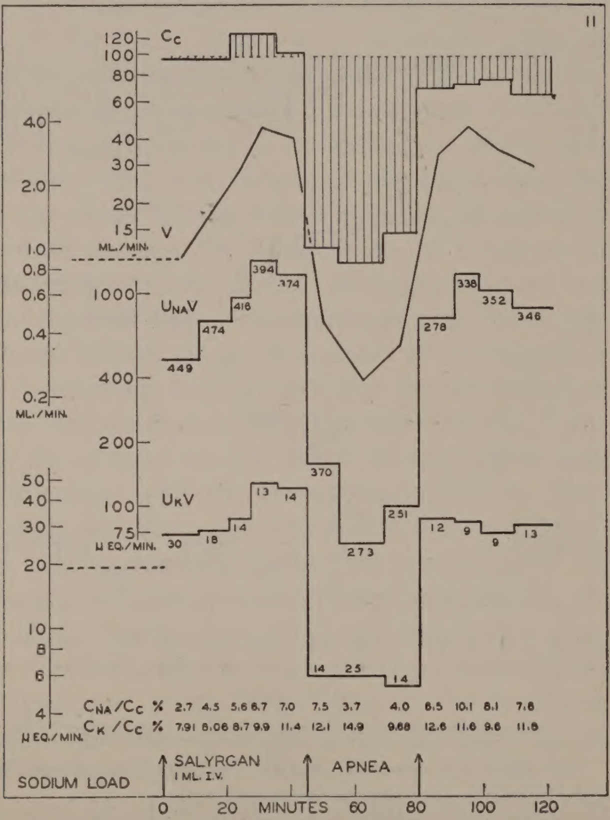

Fig. 2 The effect of a mercurial diuretic and apnea on glomerular filtration, urine flow and electrolyte excretion in the harbor seal. (Presentation and abbreviations as in fig. 1.)

Urine flow and sodium output increased sharply following administration of Salyrgan, without change in filtration. The urinary sodium concentration was high as the result of the ingestion of 10 gm of sodium chloride prior to the test and tended to fall somewhat as the urine flow increased. Potassium output increased despite a fall in urinary concentration. With apnea all values fell abruptly and then returned toward the control values during recovery. Sodium output fell much more than filtration (C_{Na}/C_c fell from 7.5 to 4.0%) but the potassium output decreased to approximately the same extent.

In every experiment, administration of the mercurial diuretic resulted in augmented urine flow and a marked increase in sodium excretion. This response was most marked after sodium chloride and water had been given by mouth. The sodium urinary concentration always rose so that sodium excretion increased more than the water output. The rise in urinary sodium concentration preceded the rise in urine flow in the experiment presented in figure 1, but the control urine flow was small and it seems likely that relative dehydration may have set in train reactions which tended to delay and minimize the diuretic effect. In the other experiments, as in that portrayed in figure 2, water (V) and sodium output ($U_{Na}V$) rose together. The values for renal excretion of potassium (U_KV) also tended to increase slightly but urinary potassium concentrations remained unchanged or fell even after preliminary loading with a potassium salt. The augmentation of water and sodium excretion occurred independently of changes in glomerular filtration. This fact is clearly shown in figures 1 and 2. In the first experiment the glomerular filtration (C_c) fell from 112 cm^3/min. to stabilize at a level of about 45 cm^3/min., a change which may be attributed to recovery from hyperemia and increased filtration induced by feeding. Despite the decrease in filtration, sodium and water output increased in response to the mercurial diuretic. In both of these and in the remainder, the sodium-creatinine (C_{Na}/C_c) and the potassium-creatinine (C_K/C_c) clearance ratios rose strikingly during mercurial diuresis indicating increased excretion of both sodium and potassium relative to filtration, presumably as a result of decreased tubular reabsorption.

Water diuresis. Although seals do not ordinarily drink water, they respond to ingested water like other mammals with a large diuresis. The efficiency of the seal's kidney in the conservation of sodium and potassium was demonstrated in the course of this response. The lowest values for urinary sodium and potassium concentrations and excretions observed in resting animals were found in 6 experiments in which water was given by stomach tube in amounts ranging from 500 to

1500 cm³. The effect of water diuresis alone was followed in one animal and water diuresis was allowed to develop during 7 and 10 periods before apnea was induced in two (fig. 3). In two the effect of pitressin was studied after control values showing the maximal effect of water loading had been obtained; one of these is presented graphically in figure 4.

The output of water may increase remarkably; in one instance it rose to 6.6 cm³/min. in association with a creatinine

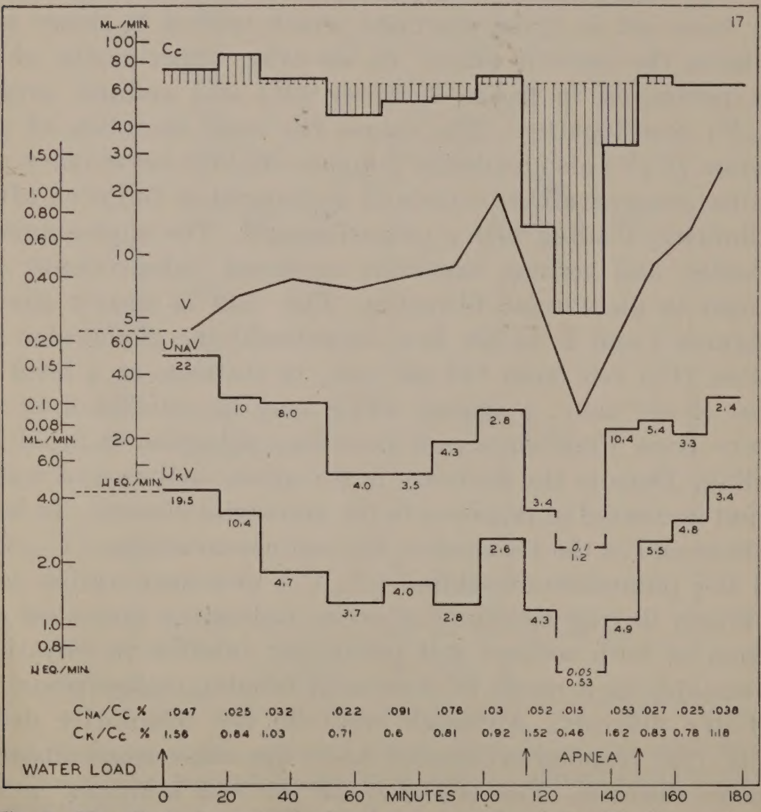

Fig. 3 The effect of a water load and apnea on glomerular filtration, urine flow and electrolyte excretion in the harbor seal. (Presentation and abbreviations as in fig. 1.)

Urine flow rose steadily over a period of two hours after 600 cm³ of water had been given by stomach tube, whereas sodium and potassium excretion decreased without change in filtration. Electrolyte output fell sharply during apnea in association with, but more than, filtration in one period.

U/P ratio of 9.0. In the experiment presented in figure 3 urine flow rose from 0.22 to nearly 1.0 cm^3/min., whereas both urinary concentration and output of sodium and potassium decreased sharply. The clearance ratios also fell, apparently

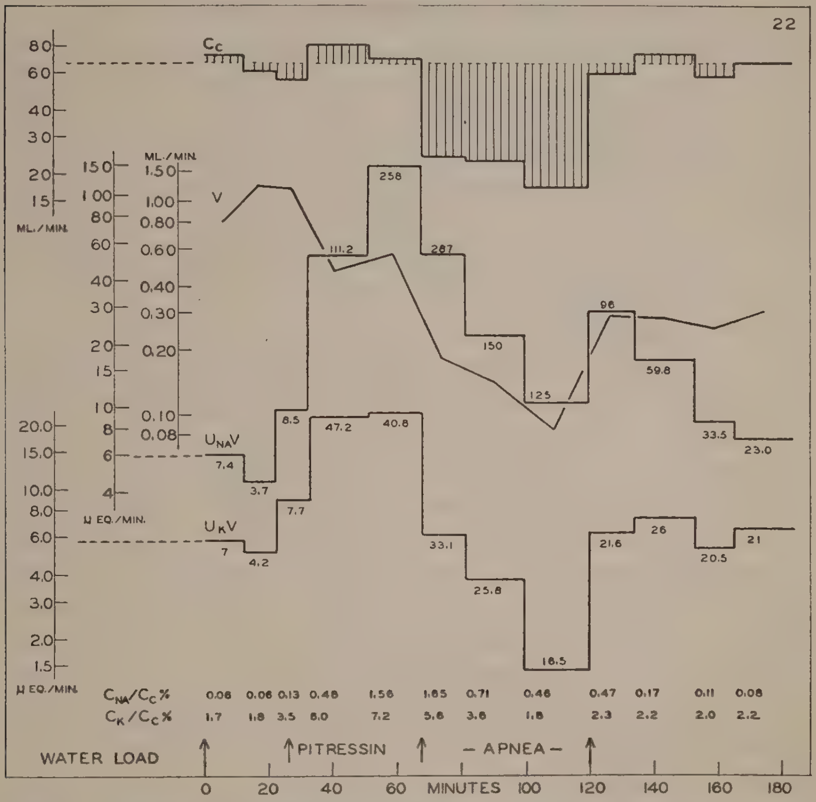

Fig. 4 The effect of pitressin and apnea during water diuresis on glomerular filtration, urine flow and electrolyte excretion in the harbor seal. (Presentation and abbreviations as in fig. 1.)

During the two control periods, a water diuresis (after 850 cm^3) had reached its peak. The associated reduction in sodium and potassium output is evident. The administration of 1000 milliunits of pitressin at 25 minutes was immediately followed by a fall in urine flow and an increase in both sodium and potassium excretion without change in filtration. Urine flow was further diminished by apnea and electrolyte output abruptly fell off in association with the decrease in filtration. The clearance ratios which had been rising under the influence of pitressin also fell off sharply indicating increased reabsorption of electrolyte. The response to apnea thus differs from that to pitressin.

because tubular reabsorption of sodium and potassium increased at a time when water reabsorption diminished. Similar low average values were observed in other experiments (16, 17, 24 — tables 1 and 2; figure 4). Water loading did not appear to influence glomerular filtration and the changes described above took place independently of any alteration in filtration. Similar results have been obtained in a study of water diuresis in the seal by Ladd and his associates (Ladd et al., '51).

The effect of pitressin. In two experiments (18, 19 — tables 1 and 2) pitressin in total doses of 700 and 1000 milliunits, respectively, was administered when the urine flow was reduced and in two others (22, 23 — tables 1 and 2; figure 4) after a water diuresis had developed. Apnea was induced in all at the height of the pitressin effect.

Little change in urine flow occurred following injection of pitressin when oliguria was present, but the urinary concentration and output of both sodium and potassium increased in the absence of change in filtration, and the clearance ratios increased. When given during a water diuresis, pitressin induced a prompt and significant reduction in urine flow (control periods — figure 4) which was associated with a striking increase in urinary concentration, excretion and clearance ratios of both sodium and potassium. Since pitressin caused no change in glomerular filtration it seems likely that the doses employed did not induce hemodynamic changes and that it acted directly upon the tubular transfer mechanisms, increasing reabsorption of water and decreasing reabsorption of sodium and potassium.

Electrolyte loading. As expected, the administration of large amounts of sodium or potassium chloride (see "Methods") resulted in elevated plasma concentration and augmented electrolyte excretion, attributable both to increased urinary concentration of sodium or potassium and to increased urine flow. These changes are apparently the result of reduced tubular reabsorption alone since glomerular filtration did not increase. Higher rates of electrolyte excretion were observed (tables 1 and 2) in those animals receiving a meal prior to study, presumably because the food contained relatively large amounts of sodium and potassium salts. There was no evidence in these data that augmented excretion of either ion influenced the ex-

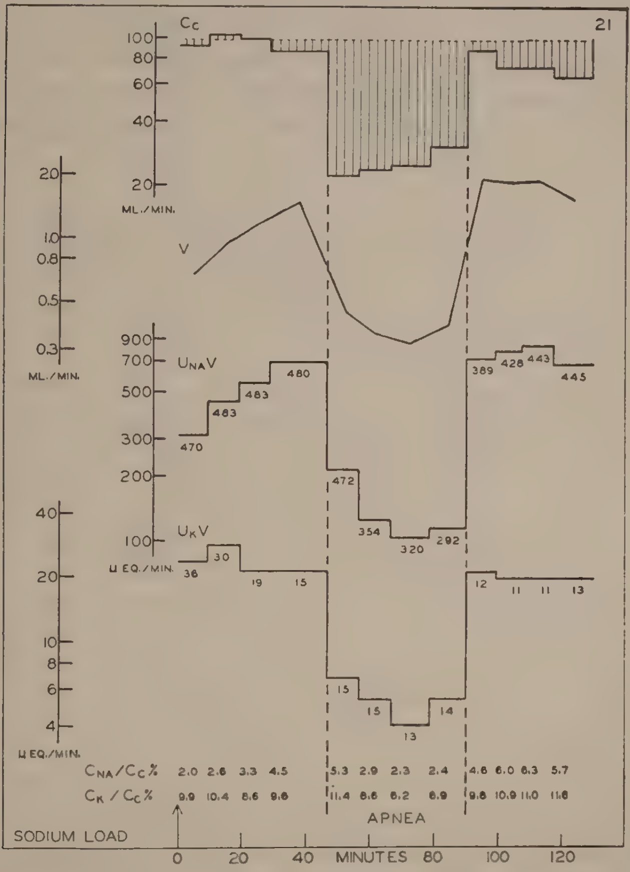

Fig. 5 The effect of sodium loading and apnea on glomerular filtration, urine flow and electrolyte excretion in the harbor seal. (Presentation and abbreviations as in fig. 1.)

Sodium chloride (10 gm) had been administered by stomach tube 60 minutes before beginning the experiment. A steadily rising sodium excretion was attributable both to a greatly increased urinary concentration of sodium (480 mEq/L) and increasing urine flow. Both glomerular filtration and potassium excretion remained constant. Apnea produced a prompt fall in all values and urinary sodium concentration diminished. Despite the need to excrete sodium the percentage of filtered sodium excreted in the urine (C_{Na}/C_c) fell from 4.5 to 2.3%, indicating increased tubular sodium reabsorption. On termination of the period of apnea all values tend to return to the control level.

cretion of the other, though this point was not subjected to a critical examination (see figs. 5 and 6).

As much as 8% of sodium ion and 60% of potassium ion filtered at the glomerulus was excreted in the urine after loading. There was no evidence of tubular secretion of potassium resembling that observed in the dog (Berliner and Kennedy, '48; Mudge et al., '48), but more thorough preparation of the seal may be necessary before tubular secretion of potassium is

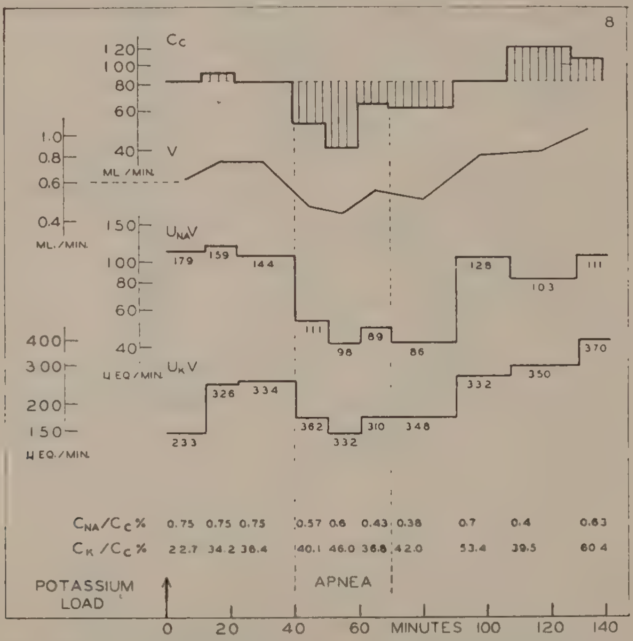

Fig. 6 The effect of potassium loading and apnea on glomerular filtration, urine flow and electrolyte excretion in the harbor seal. (Presentation and abbreviations as in fig. 1.)

Potassium chloride (10 gm) had been given by stomach tube 45 minutes prior to study and in doses of 15 gm daily for three days previously. Potassium excretion was greatly increased, chiefly because the urinary concentration was high (334 mEq/L) and urine flow was somewhat higher than usual. Sodium output and glomerular filtration remained constant throughout the control period. With apnea all values decreased; potassium output changed to approximately the same extent as filtration, but sodium output fell more. On termination of apnea sodium output and filtration returned to control levels, whereas potassium and water output climbed steadily, well above control values. Indeed, the amount of potassium excreted came to as much as 60.4% of the filtered load (C_K/C_c).

detectable. The urine flow always rose with the sodium or potassium output presumably as a result of osmotic diuresis. Since no change in filtration was noted it may be concluded that the alteration in excretion was entirely attributable to altered tubular activity.

The effect of filtration changes on water and electrolyte excretion. As noted in earlier studies (Hiatt and Hiatt, '42) feeding was followed by an increase, and apnea by a decrease in glomerular filtration rate. The remarkable range in filtration thus made possible (from 180 to less than 10 cm^3/min.) revealed a close correlation between filtration rate and the excretion of sodium, potassium and water. An almost exact linear correlation seems to obtain between filtration and reabsorption in dog and man (Smith, '51) though correlation is more apparent than real, owing to the very small percentage of filtered load which escapes reabsorption. The relationship between filtration and excretion, on the other hand, is for the same reason by no means so obvious in these animals as it becomes in the seal.

The effects of the abrupt and large decrement in filtration during apnea upon sodium, potassium and water excretion are evident in the data tabulated in tables 1, 2 and 3. These figures are averages of two or more clearance periods obtained before, during and after apnea. As averages, they are of course somewhat misleading since the effect of the change is minimized by the inclusion of periods collected shortly after apnea was initiated in which the washout of urine formed in the control period and held in the renal "dead space" (tubules, collecting ducts, renal pelves and ureters) probably gave rise to falsely high values. Concentrations in the first sample of urine collected after apnea were often low for similar reasons. On several occasions (see tables 1 and 2) the effect of apnea was altogether masked. Despite this difficulty, it was deemed proper to treat the data throughout in a uniform manner. It should be emphasized that error introduced in this way obscured or minimized the observed effects and did not exaggerate them.

TABLE 1

Effect of apnea on sodium excretion[1]

EXPERIMENT	CONTROL			APNEA			RECOVERY			COMMENT
	U_{Na}	$U_{Na}V$	C_{Na}/C_{cr}	U_{Na}	$U_{Na}V$	C_{Na}/C_{cr}	U_{Na}	$U_{Na}V$	C_{Na}/C_{cr}	
	mEq/L	*uEq/min*	%	*mEq/L*	*uEq/min*	%	*mEq/L*	*uEq/min*	%	
20	52.3	9.8	0.11	36.0	3.3	0.10	43.2	6.1	0.08	Fasting
3	324.0	155.4	0.56	158.5	45.7	0.40	228.5	90.2	0.67	Fed 45 to 60 minutes prior to study
4	360.7	314.0	1.78	246.5	124.0	0.99	278.0	170.3	1.43	
9	....	....	...	126.6	34.0	0.35	201.2	84.5	0.81	
6	246.0	125.9	0.75	206.0	26.3	1.16	287.0	266.7	2.85	Salyrgan (1 ml) prior to apnea
10	225.3	90.2	0.73	200.0	13.0	0.30	270.0	127.1	1.31	Salyrgan (1 ml) p.t.c.
11	394.6	1207.0	6.44	299.6	107.5	5.06	328.5	967.0	8.10	NaCl load — Salyrgan p.t.c.
13	....	....	...	165.0	23.1	0.59	339.3	318.0	2.73	NaCl load — fasting
14	457.3	371.0	2.68	363.6	89.1	2.04	...	...	...	NaCl load — fed 60 min. p.t.c.
21	480.7	500.2	3.09	359.5	139.4	3.16	426.2	750.2	5.61	NaCl load — water load
8	160.5	112.6	0.75	99.3	47.6	0.53	105.6	75.6	0.50	KCl load
12	385.3	366.9	2.65	269.5	53.1	2.76	287.0	239.0	2.07	KCl load — Salyrgan p.t.c.
16	8.5	3.4	0.04	24.8	2.8	0.16	...	...	...	Water load
17	3.5	2.0	0.03	5.0	1.1	0.04	3.7	2.5	0.03	Water load
24	1.6	9.7	0.12	7.4	2.1	0.19	...	...	...	Water load
18	226.0	59.0	0.53	148.3	14.4	0.38	...	...	...	Pitressin 400 milliunits
19	126.2	29.8	0.30	25.5	2.7	0.06	98.5	21.5	0.19	Pitressin 1000 milliunits
22	184.6	98.8	1.02	187.3	28.5	0.94	63.1	17.7	0.25	Pitressin 1000 mU, water load
23	103.6	33.7	0.26	173.2	34.6	0.62	152.1	32.4	0.39	Pitressin 1000 mU, water load

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[1] All values are averages of figures obtained in two or more consecutive urine collection periods except during apnea in experiment 13.

Abbreviations are as follows: U_{Na} = urinary concentration of sodium ion; $U_{Na}V$ = urinary output of sodium ion; C_{Na}/C_{cr} = sodium-creatinine clearance ratio, or the percentage of filtered sodium ion appearing in the urine each minute; p.t.c. = prior to control period.

TABLE 2

Effect of apnea on potassium excretion[1]

EXPERIMENT	CONTROL			APNEA			RECOVERY		
	U_K	U_KV	C_K/C_{cr}	U_K	U_KV	C_K/C_{cr}	U_K	U_KV	C_K/C_{cr}
	mEq/L	*uEq/min*	%	*mEq/L*	*uEq/min*	%	*mEq/L*	*uEq/min*	%
20	9.5	1.7	0.68	6.6	0.6	0.63	11.0	1.5	0.85
3	80.8	38.7	4.95	78.2	23.6	7.17	64.6	25.9	6.50
4	25.0	22.0	5.47	33.6	16.9	5.07	22.0	13.4	4.86
9	. .	. . .	. . .	23.7	6.3	4.20	35.0	14.4	7.80
6	44.3	23.2	5.54	46.3	6.1	11.60	19.2	18.6	8.98
10	37.9	15.0	4.40	44.9	3.0	5.53	44.8	20.6	7.80
11	13.6	41.6	10.00	17.5	5.7	12.25	10.6	30.4	11.39
13	. .	. . .	. . . .	61.0	8.5	7.06	18.6	17.0	5.16
14	29.8	24.3	6.75	37.2	10.1	8.11	. .		.
21	25.2	24.0	9.60	14.1	5.4	8.35	11.9	20.8	10.84
8	297.0	213.3	31.06	334.6	161.2	40.90	343.2	243.2	45.0
12	130.0	122.3	22.89	95.2	19.4	25.60	142.2	119.2	28.7
16	4.0	1.5	0.65	6.6	0.7	1.39		. .	. . .
17	3.1	1.8	0.78	3.2	0.8	1.22	4.6	3.3	0.93
24	1.4	8.5	4.91	4.9	1.4	7.49	.	.	. . .
18	20.9	5.7	. . .	12.6	1.2	. . .	. .	.	. . .
19	30.2	7.1	3.34	9.5	1.0	1.08	40.5	8.4	3.84
22	44.0	15.5	6.60	25.1	10.9	3.64	22.7	5.0	2.17
23	56.1	18.6	3.45	29.7	5.8	2.91	37.6	8.2	4.09

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[1] Abbreviations are as follows: U_K = urinary concentration of potassium ion; U_KV = urinary output of potassium ion; C_K/C_{cr} = potassium-creatinine clearance ratio, or the percentage of filtered potassium appearing in the urine each minute. See table 1 for comment.

The average values for urinary sodium concentration, sodium excretion, and sodium-creatinine clearance ratios before, during, and after apnea are presented in table 1. In two experiments apnea was induced at the outset and control values were not obtained (9, 13 — table 1). However, the values after apnea during the recovery period clearly show the changes produced. On 4 occasions measurements were not made during recovery. Figures for potassium excretion during the same experiments are presented in table 2. The complete data of 5 experiments are presented graphically in figures 2 to 6.

Regardless of preliminary treatment, the sodium excretion (table 1) always fell markedly in association with the reduction in filtration induced by apnea. The urinary sodium concentration likewise decreased in most of these experiments, including those in which control sodium output had already decreased as the result of water diuresis. The clearance ratio tended to fall, indicating greater depression in sodium excretion than in glomerular filtration, presumably as a consequence of a relative increase in tubular sodium reabsorption. This phenomenon was obscured or prevented by preliminary water loading, even if diuresis had been halted by administration of pitressin (19 and 22 — table 1).

Apnea had a less marked but similar effect on potassium excretion. Here, however, the decrease in output was somewhat less than the fall in filtration on the average, so that the potassium-creatinine clearance ratio tended to rise. The clearance ratio decreased significantly only during pitressin antidiuresis (19, 22, 23 — table 2 and fig. 4). There was also evidence of this effect in potassium-loaded seals, obscured in the average values (8, 12 — table 2) but demonstrable in the detailed data (fig. 6).

Urine flow usually decreased greatly during apnea but not to the same extent as glomerular filtration. As a result the creatinine U/P ratio usually decreased (table 3). In 15 of 17 experiments in which control values had been obtained and a relatively steady physiologic state maintained, the creatinine U/P ratio fell or did not change significantly. In three studies

TABLE 3

Effect of apnea on filtration rate, urine flow and creatinine urine-plasma concentration ratio[1]

EXPERIMENT NUMBER	CONTROL			APNEA			APNEA/CONTROL CONC. RATIO (1)/(2)	COMMENT
	C_{cr}	V	U/P_{cr} (1)	C_{cr}	V	U/P_{cr} (2)		
	ml/min	*ml/min*	*times*	*ml/min*	*ml/min*	*times*	%	
26	84	1.77	48	47	1.68	28	58	Glucose diuresis
27	145	2.58	64	10	0.20	49	77	
29	150	2.20	68	53	1.42	37	55	
20	61	0.18	331	23	0.09	247	75	Fasting
3	177	0.49	372	61	0.30	194	52	Fed
4	106	0.87	123	74	0.57	124	101	
10	75	0.40	191	16	0.07	208	109	Mercurial diuresis
11	109	3.08	36	12	0.35	36	100	NaCl load
14	84	0.81	104	20	0.18	107	103	
8	85	0.71	121	52	0.48	107	89	KCl load
12	81	0.95	86	11	0.19	56	65	
16	50	0.45	115	10	0.11	92	80	Water diuresis
24	65	6.00	11	10	0.28	39	355	
18	74	0.26	283	25	0.10	255	90	Pitressin
19	72	0.23	306	25	0.08	282	92	
22	75	0.51	147	21	0.14	163	111	Pitressin and water load
23	90	0.30	293	41	0.20	216	73	

[1] All values are averages of two or more consecutive clearance figures. Experiments 26, 27 and 29 were studies of diuresis during maintenance of high plasma glucose concentrations by intravenous glucose infusion; electrolyte concentrations were not measured. Experiments 9 and 13 (tables 1 and 2) have been omitted because control periods were not obtained; 6, because mercurial diuretic was administered just before apnea was induced; 21 and 17, because the values were changing so rapidly prior to apnea that satisfactory control averages could not be computed. Abbreviations are as follows: C_{cr} = creatinine clearance; V = urine flow; U/P_{cr} = urine-plasma concentration ratio. See table 1 for comment.

made during an osmotic diuresis (glucose loading) the creatinine U/P ratio fell to as low as 55% of the control value. Preliminary treatment with pitressin minimized but did not abolish the effect in experiments 18, 19, 22, 23 (table 3); the U/P ratio rose significantly only in apnea during water diuresis (24 — table 3). Sodium loading and mercurial diuresis (10, 11, 14 — table 3) appeared to reduce the magnitude of the response.

DISCUSSION

The present study has revealed no unique renal water- or electrolyte-handling mechanism in the diving mammal. Major changes in water or electrolyte excretion may be produced by changes in filtration. Under standard, more or less steady, conditions, however, the kidney of the seal is capable of remarkable excretory variability independent of glomerular filtration. Loading with sodium or potassium salts or with water results in increments in sodium, potassium or water excretion, respectively, without change in filtration. Diuresis may be induced by a mercurial diuretic, or urine flow may be abruptly suppressed by pitressin, almost entirely as a result of changes in tubular activity. When filtration is greatly reduced by apnea or increased after feeding, the tubular mechanisms ordinarily controlling urinary output are no longer effective, and water and electrolyte excretion then tend to follow filtration. Indeed, with low filtration tubular activity may fail to operate in accord with body needs, i.e., water and salt retention occurs despite the need to eliminate loads imposed experimentally.

Measurements of tubular mass in terms of the maximal glucose reabsorption rate (glucose Tm) have not been made during apnea but the Hiatts (Hiatt and Hiatt, '42) have found that glucose Tm remained unchanged during feeding hyperemia, indicating that large variations in filtration may occur without changes in the number of active glomeruli. The intra-renal vasoconstrictive response to apnea appears to involve both afferent and efferent arterioles (Bradley and Bing, '42) in a relatively uniform manner. Although filtration may cease in some glomeruli, it seems likely that filtration decreases in all the residual active units. In this view, filtrate must perfuse tubules at a slower rate than normal, resulting in a longer period of contact between glomerular filtrate and reabsorbing tubular cells. Moreover, the load brought to the tubules is reduced and the reabsorptive mechanisms may operate relatively more effectively in removing some solutes from the filtrate, thus accounting for the greater reduction in the output of sodium than in filtration. Elevated renal venous pressure also results in augmented sodium reabsorption, and cannot be eliminated as a contributory factor (Blake et al., '49). Potassium excretion seldom fell more than the filtration rate during apnea and potassium loading had no effect on sodium excretion indicating that the reabsorption of potassium is handled by a different tubular cellular mechanism which is more resistant to changes in filtration. Possibly potassium secretion accounts in part for this apparent independence.

Electrolyte and water reabsorption are probably iso-osmotic in the proximal tubular segment and interdependent; whereas, electrolyte and water reabsorption go on independently in the distal segment, hypotonic or hypertonic urine being formed as needs demand (Smith, '51). For this reason it might have been anticipated that water and electrolyte excretion would decrease in parallel during apnea and that water reabsorption would also increase relative to filtration. The observed reduction in water reabsorption cannot be attributed to diminished secretion of ADH by the posterior pituitary, because it occurred despite preliminary administration of pitressin and because increased ADH production apparently could account for an elevation in water reabsorption during apnea imposed in the course of water diuresis. Since sodium loading (but not potassium) and mercurial diuresis seemed to permit augmented reabsorption of water it may be surmised that the resulting elevation in sodium load in the tubular urine entering the distal segment might have been important in permitting water reabsorption under the influence of ADH. In this view, the action of ADH in the distal segment may require a minimal load or concentration of sodium in the tubular urine that is not achieved during apnea as a result of a relative increment in proximal segment reabsorption. It is possible that hypoxia during apnea may have a detrimental effect upon distal tubular mechanisms for water reabsorption.

These observations have relevance to the problems of the adaptation of the seal to its environment. Further evidence has been adduced to show that the seal cannot use sea-water as a source of water. Under large sodium loads in these experiments the seals excreted urine containing little more sodium than sea-water. Drinking sea-water could result in no net gain and probably would induce a negative water balance. However, the remarkable reactivity of the phocine kidney and the salvage of electrolyte and water permissible at low filtration rates provides the seal with an efficient system for making the best use of the scanty water supply available. As the Hiatts have indicated, post-prandial hyperfiltration allows excretion of excess water and, as these studies reveal, of excess potassium and sodium taken with the food so that dangerous plasma concentrations of these substances do not develop. In one seal treated with daily doses of potassium chloride, death as a result of potassium poisoning apparently occurred, in large part because the animal refused food and was thus deprived of the beneficial operation of post-prandial hyperfiltration in eliminating excess salt.

SUMMARY

The renal excretion of sodium, potassium and water has been measured in 6 young female seals (Phoca vitulina L.) in relation to glomerular filtration rate. Electrolyte and water excretion varied widely in response to loading, mercurial diuresis and pitressin anti-diuresis, independently of filtration. However, the marked diminution in glomerular filtration during apnea has been found to reduce the output of electrolytes and water regardless of need.

Urine flow increased markedly following ingestion of 500 to 1500 cm^3 of water, whereas sodium and potassium output diminished. Pitressin (800 to 1000 milliunits intravenously and

subcutaneously in divided doses) induced a prompt reduction in urine flow associated with augmented sodium and potassium excretion. The administration of sodium chloride (10 to 20 gm by mouth) was followed by increased sodium and water output without change in potassium excretion. Likewise loading with potassium chloride (10 to 20 gm by mouth) failed to alter sodium excretion although the output of potassium and water rose. These changes occurred without any consistent alteration in glomerular filtration.

Sodium, potassium and water excretion fell sharply with the fall in filtration during apnea, regardless of the state of hydration or body electrolytes. Sodium output usually fell more, and urine flow less than filtration. The potassium output tended to change proportionately.

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THE BINDING OF COPPER BY HEMOCYANIN

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TWO FIGURES

Many marine animals accumulate remarkably large amounts of copper from sea water, producing a concentration of copper within their bodies far exceeding that in their external environment (Redfield, '34). The horseshoe crab, *Limulus polyphemus,* and the sea conch, *Busycon canaliculatum,* are among the marine animals which can accomplish this. They produce blue-colored respiratory pigments, known as hemocyanins; these carry nearly all of the copper found in their bodies.

Kobert (Kobert, '03) first observed that the addition of cyanide to oxygenated hemocyanin (oxyhemocyanin) results in the release of oxygen, and the decolorization of the protein. Kubowitz ('38) showed that this decolorization was due to the ability of the cyanide to form a complex with the copper ions, removing them from the protein. Previous to the work of Kubowitz, Pearson ('36) had attempted to explain the decolorization by postulating the formation of a protein-cyanide complex, or cyano-hemocyanin. An equilibrium constant was calculated for the postulated reaction. In view of Kubowitz's discoveries, it seems reasonable to calculate instead an equilibrium constant for the dissociation of copper from hemocyanin.

Such a constant has been calculated from a series of experiments in which cyanide, which has a known affinity for cuprous ion, is allowed to compete with the protein for the metal.

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METHODS

The unpurified sera of both *Limulus polyphemus* and *Busycon canaliculatum* were used in these experiments, since the protein consists in each case of at least 90% pure hemocyanin (Redfield, '28 and Montgomery, '30). The serum was obtained by allowing the whole blood to stand until the white cells had coagulated. The clear blue solution was then poured off and filtered. Copper analysis was performed colorimetrically. Three milliliters aliquots of the serum were wet-ashed with concentrated sulfuric acid and Superoxol. Color was developed in the resultant solution by the addition of sodium diethyl dithiocarbamate, using a slight modification of a standard procedure (Price and Telford, '50). The method gave reproducibility of better than 0.5%.

The oxyhemocyanin concentration was determined spectrophotometrically (Pearson, '36). Measurements of the *Limulus* serum were made at 590 mμ, and of *Busycon,* at 570 mμ. A Beckman model DU Quartz Spectrophotometer was used. pH measurements were made with a Beckman Model G pH meter, with use of a dipping glass electrode. The cyanide solutions were standardized against silver nitrate solution of known concentration (Kolthoff and Sandell, '48).

In each set of experiments, a constant amount of serum was mixed with increasing quantities of potassium cyanide solution, and made up to volume with either buffer or water. The mixtures were allowed to stand, stoppered, at room temperature, until no further change could be observed in the extinction coefficient (generally 24 hours at 25–28°C.). The solutions were centrifuged before being read in the spectrophotometer.

THEORETICAL

It is easy to see, qualitatively, that the binding of the copper to the protein must be relatively strong. Pearson found that a large excess of cyanide over copper had to be added to produce complete decolorization. We found that the addition of 100 moles of imidazole per mole of copper produces no decolorization, although imidazole has an affinity for copper as strong as that of ammonia (Gurd, Edsall, Felsenfeld and Goodman, '52).

The complexity of the protein molecule makes it necessary to simplify the picture of this competition as much as possible. The protein molecule is assumed to consist of a number of identical reactive groups or sites, denoted below by the letter R, each of which is capable of binding a maximum of two copper ions. The assumption is made that pairs of copper ions on the same site interact, but that copper ions on different sites behave independently. This model for the protein molecule seems reasonable in view of the one-to-one ratio of oxygen atoms to copper atoms in oxyhemocyanin. It has been suggested that an oxygen molecule "bridges" a pair of copper atoms (Rawlinson, '41). This would require that pairs of copper atoms be situated close together in the molecule, leading to a much stronger interaction between the pair of copper atoms of any given site than between those belonging to different sites. It has been shown[1] that if copper ion in excess is added to native hemocyanin, it will be bound only weakly by the protein. This indicates that in hemocyanin, the binding sites discussed in this paper are saturated with copper.

The approximation is made throughout this paper that concentration and activity are equal. The reactions which are assumed to occur, and their corresponding equilibrium constants, are:

$$RCu_2O_2 = RCu_2 + O_2 \qquad K_o = \frac{(RCu_2)\,(O_2)}{(RCu_2O_2)} \qquad (1)$$

$$RCu_2 = RCu + Cu^+ \qquad k_2 = \frac{(RCu_2)}{(Cu^+)\,(RCu)} \qquad (2)$$

$$RCu = R + Cu^+ \qquad k_1 = \frac{(RCu)}{(R)\,(Cu^+)} \qquad (3)$$

$$Cu^+ + 2CN^- = Cu(CN)_2^- \qquad k_B = \frac{(Cu^+)\,(CN^-)^2}{(Cu(CN)_2^-)} \qquad (4)$$

The first assumption made is that the copper in reduced hemocyanin is in the form of cuprous ion. Kubowitz's experiment provides convincing evidence for this. He was able to reactivate protein from which copper had been removed, only by the addition of cuprous ion, and not by the addition of cupric ion. Hemocyanin, in the deoxygenated state, completely lacks the blue color characteristic of cupric complexes. It should be noted that no assumption is made about the oxidation state of copper in oxyhemocyanin.

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[1] I. Klotz, personal communication.

The concentration of reduced hemocyanin, RCu_2, was calculated from the measured concentration of oxyhemocyanin, RCu_2O_2. The constant K_o used in these calculations was taken from previously published data on oxygenation (Redfield, '30, and Redfield and Ingalls, '33). In each case a single constant was used to describe the oxygenation curve. This was necessary because the pairs of constants given by Redfield and Ingalls only fit the curve at relatively low oxygen pressures, where there is considerable deviation from hyperbolic form. At higher pressures, at which the various curves probably coincide quite closely with the hyperbolic case, the constant for the hyperbolic curve was used. This seemed to be justified by the reasonably good consistency of the results obtained from this method and those from experiments with deoxygenated serum, where the oxygen constant was not involved in the calculation.

Equations 2 and 3 assume that the copper dissociates from the unoxygenated hemocyanin only, and that we may neglect the possible existence of the species RO_2. This assumption was justified by an experiment which showed that the amount of copper removed from the protein depends upon the concentration of unoxygenated hemocyanin. In this experiment, concentrations of reduced hemocyanin were increased by the reduction of the partial pressure of oxygen. By the use of a water aspirator, oxygen was removed from samples of serum placed in small suction flasks. A few drops of capryl alcohol were added to prevent foaming. When maximum decolorization had been achieved, the flasks were filled with nitrogen, and the desired quantity of cyanide solution added through the nitrogen atmosphere. The flasks were quickly re-evacuated, and allowed to stand in such a condition for several hours, until the final readings to be described indicated that equilibrium had been reached.[2] When air was readmitted, some blue color returned, but the extinction coefficient was much lower than that obtained by performing the same experiment in air, using the same quantities of serum and cyanide. The color intensity increased very slowly on exposure to air (a few days for detectable changes), and it was therefore possible to use the extinction coefficient as an indication of the equilibrium position in vacuum, assuming that the introduction of oxygen disturbed the equilibrium extremely slowly. The fact that decolorization is so much greater when large quantities of reduced hemocyanin are present indicates that the loss of oxygen must involve a considerable decrease in the copper binding constant — a phenomenon which seems to be quite similar to one observed by Joselow and Dawson ('51) for the copper enzyme ascorbic acid oxidase. This decrease is, in fact, demanded by thermodynamic considerations if the oxygen-carrying properties of the protein are completely destroyed by removal of copper.

The final assumption concerning the copper dissociation, that $k_2 >> k_1$, is equivalent to saying that the concentration of the species RCu, where the copper exists alone on a given site, is negligible in the first approximation. Such a postulate leads to the calculation of two association constants, with $k_2 >> k_1$, so that the assumption leads to a consistent answer. No other assumption yields a consistent conclusion, since the data is more than sufficient to determine the two constants uniquely.

The copper-cyanide reaction is assumed to go as far as the formation of the double cyanide complex, but no further. Where large excesses of cyanide were present, allowance for the formation of the triple complex did not make significant changes in the calculated results. The value used for the constant, $k_B = 10^{-23}$, is the one given by Bjerrum ('50). Its somewhat doubtful accuracy is one of the limiting factors in the accuracy of the results obtained. Because of the inaccuracy of K_o and k_B, and the errors of measurement, the error of the constants calculated in this paper is probably of the order of ± 1.0 in logarithms.

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[2] Equilibrium was attained much more rapidly under these conditions than it was when the experiments were performed in air. The relative rapidity of the initial rate of dissociation, compared with the rate of recombination of copper and protein, suggests that the dissociation rate must slow down considerably when the equilibrium point has been nearly reached.

Using the treatment of Scatchard ('49), Klotz ('46), and others, we first define [3] a quantity ν,

$$\nu = \frac{\text{copper bound to reduced hemocyanin}}{\text{total sites on reduced hemocyanin}}$$

ν has a maximum value of 2. For these experiments,

$$\nu = \frac{2 \times (RCu_2)}{(R) + (RCu_2)}$$

The intrinsic constant for the copper-protein reaction (the reaction of copper with one of the two "spots" on a given site, regardless of whether the other "spots" are occupied), is given by

$$k = \frac{\nu}{(2 - \nu)\,(Cu^+)}$$

If log k is plotted against ν, extrapolation to $\nu = 0$ will give the logarithm of the first constant, while extrapolation to $\nu = 2$ will give the logarithm of the second constant. These must be statistically corrected (here, by adding and subtracting log 2 for k_1 and k_2, respectively), to give the true constants defined in equations 2 and 3. The method is therefore as follows:

1. The concentration of $RCu_2\,O_2$ is calculated from spectrophotometric data.
2. The concentration of RCu_2 is calculated from the oxygenation constant.
3. The concentration of R is found knowing $RCu_2\,O_2$, RCu_2, and the total copper in the protein.
4. The concentration of $Cu\,(CN)_2^-$ is calculated from the quantity of R present.
5. The concentration of CN^- is calculated from the total cyanide added, the value of $Cu\,(CN)_2^-$, the value for the dissociation constant for HCN, 7.2×10^{-10}, and the pH.

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[3] The symbol ν is equivalent to the symbol $\bar{\nu}$ of Scatchard ('49).

6. ν, Cu^+ and k may then be calculated from the above formulas. When the vacuum-nitrogen route is used, it is possible to measure the concentration of RCu_2 directly. It is equal to the amount of oxyhemocyanin formed when oxygen is admitted.

7. k_1 and k_2 are calculated from k as described above.

RESULTS

Table 1 gives the results of the first series of experiments on cyanide competition in air. Two milliliter samples of *Limulus* serum were treated with varying amounts of KCN solution, and the mixtures made up to a total volume of 12 ml with borate buffer. The buffer is made by the addition of solid sodium hydroxide to a 0.1 M solution of H_3BO_3, to give a pH of 8.31. In all of the experiments described in this paper, the partial pressure of oxygen was taken as 159.5 mm of mercury and the oxygenation constant K_o for *Limulus* hemocyanin, was taken equal to 0.32 for pH greater than 8, and as 0.40 for pH equal to or less than 8 (Redfield, '30).

TABLE 1

Limulus serum, buffered in the region of pH 8.1
KCN concentration: 7.12 × 10^{-3} M.
Copper concentration in all final solutions: 0.388 mM.

KCN CC. ADDED	OXYHEMOCYANIN %	pH	ν	LOG k
0	100.0	7.90	...	....
1.00	99.0	7.96	...	....
2.00	94.0	8.00	0.392	18.91
3.00	80.4	8.02	0.158	18.17
4.00	62.8	8.07	6.60×10^{-2}	17.80
5.00	37.2	8.10	2.31×10^{-2}	17.33
6.00	19.1	8.12	9.24×10^{-3}	16.97
7.00	8.0	8.17	3.42×10^{-3}	16.95
8.00	6.5	8.23	2.73×10^{-3}	16.86

Extrapolation of the curve to $\nu = 0$ (fig. 1, solid circles) gives for the association of the first copper ion an intrinsic constant (i.e., statistically uncorrected) of $10^{16.7}$, and a corrected constant, k_1, of $10^{17.0}$. This curve does not permit extrapolation to $\nu = 2$, so that it is impossible to determine k_2 directly from the data of table 1.

Table 2 shows the results of a similar experiment with *Busycon* serum. Four milliliter samples of serum were used, and made up to a total volume of 12 ml with KCN and the borate buffer described above. The oxygenation constant was

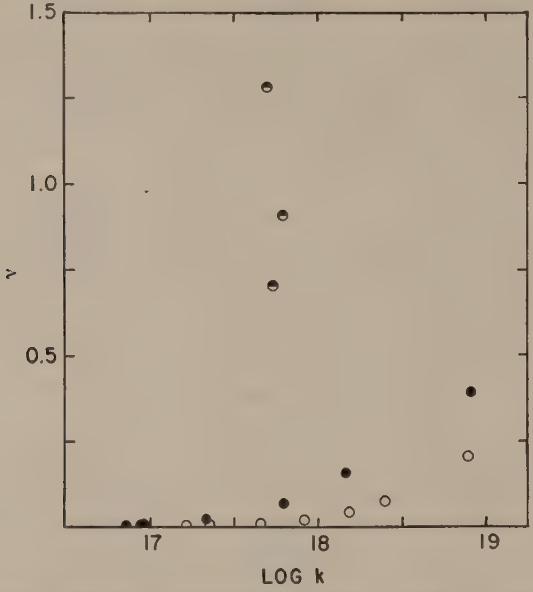

Fig. 1 Intrinsic constants, uncorrected for proton affinity. Legend: pH 8.1 ●; pH 8.8 ○; vacuum ◓.

TABLE 2

Busycon serum, buffered in the region of pH 8.1
KCN concentration: 7.12 × 10^{-3} M.
Copper concentration in all final solutions: 0.501 mM.

KCN CC. ADDED	OXYHEMOCYANIN %	pH	ν	LOG k
0	100.0	7.96	. . .	. . . .
1.00	86.7	8.03	0.118	17.01
2.00	62.7	8.08	3.02×10^{-2}	16.47
2.50	58.8	8.08	2.56×10^{-3}	16.61
3.00	48.5	8.11	1.69×10^{-2}	16.60
3.50	40.8	8.13	1.25×10^{-2}	16.56
4.00	36.9	8.15	1.06×10^{-2}	16.64
5.00	33.9	8.21	9.28×10^{-3}	16.91

assumed to be 0.70 (Redfield and Ingalls, '33). A value of $10^{16.8}$ at pH 8.3 was obtained for k_1 in *Busycon* hemocyanin, indicating that there is no striking difference between the behavior of the *Busycon* hemocyanin and that of the *Limulus*.

A somewhat different result was obtained from *Limulus* serum when the pH was increased to 8.7. In this experiment, a borate buffer adjusted to a pH of 8.88 was used. The KCN solution was diluted 1:1 with this buffer, and the resultant cyanide solution added to 1 ml portions of the serum. The mixtures were made up to a total volume of 12 ml with a solution of the original borate buffer diluted 1:1 with water. The results, shown in table 3 and figure 1, open circles, give a value for the true k_1 at pH 8.7 of $10^{17.4}$, a significant increase over the value obtained at pH 8.0. This increase is significant even though the estimated error for the final answer is considerably larger, since the major part of the error is introduced through the uncertainty in choice of constants mentioned above.

TABLE 3

Limulus serum, buffered in the region of pH 8.7
KCN concentration (buffered): 3.56×10^{-3} M.
Copper concentration in all final solutions: 0.194 mM.

KCN CC. ADDED	OXYHEMOCYANIN %	pH	V	LOG k
0	100.0	8.69	...	....
1.00	97.5	8.69	...	....
2.00	83.1	8.70	0.203	18.89
3.00	65.3	8.70	7.47×10^{-2}	18.39
4.00	49.2	8.71	3.89×10^{-2}	18.18
5.00	33.1	8.72	1.97×10^{-2}	17.92
6.00	17.8	8.73	8.58×10^{-3}	17.66
7.00	7.6	8.76	3.24×10^{-3}	17.36
8.00	4.2	8.77	1.71×10^{-3}	17.22

The change in the value of k_1 with increasing pH suggests that the group on the protein which binds the copper ion is capable of binding hydrogen ion. An amino, sulfhydryl, or imidazole group, among others, would behave in such a manner. In the calculations made above, it has been assumed that each site, R, which had no copper ion attached to it, would be capable of recombining with copper ion. One must now take into account the existence of another species, RH^+, which reduces the amount of free R available for recombination. This results in competition between hydrogen and copper ions for a place on the protein.

The correction for hydrogen ion dissociation requires a more detailed specification of the protein model. If, for example, a single group, such as a sulfhydryl group, holds each copper ion, then the species RH^+ and RH_2^{++} are possible; if a pair of amino groups holds each copper ion, then all of the species from RH^+ to RH_4^{++++} might exist. Many such models were tried. It was found that none gave better results in the procedure to be described than the simple model in which only one hydrogen ion could be attached to each site. Such behavior is consistent with models of the sulfhydryl type, provided that the acid association constant for the formation of RH^+ is much larger than the constant for the formation of RH_2^{++}, so that the concentration of the diprotic species is negligible. This is usually true for a dibasic acid in which the sites of bound hydrogen are fairly close together.

By assuming the existence of a species RH^+, and trying different values for an acid dissociation constant,

$$k_A = \frac{(R)\ (H^+)}{(RH^+)}$$

one can estimate a value for k_A, since the new copper constant k_1' should be independent of pH when a proper value of k_A is chosen. By such a procedure, it was found that a value of pk_A of about 10.0 allows the data of tables 1 and 3 to be reinterpreted so that the curves now extrapolate (fig. 2) to a common point at $\nu = 0$. From these curves one finds a statistically corrected value for k_1 of $10^{18.7}$, and for k_2, of $10^{20.5}$. This is in agreement with our assumption that $k_2 >> k_1$. A theoretical curve, using these values, has been drawn for comparison. It is not possible to make the two sets of experimental points, or the theoretical curve, coincide entirely by the choice of a single acid constant, very possibly due to electrostatic corrections which are not made in this paper, and which might become more important as more charged copper ion is attached to the protein (i.e., for large ν). No explanation is offered here for the apparent positive interaction in the complexing of the second copper. It may be, in part, the result of the choice of the model for copper dissociation (i.e., pairs of copper ions interacting).

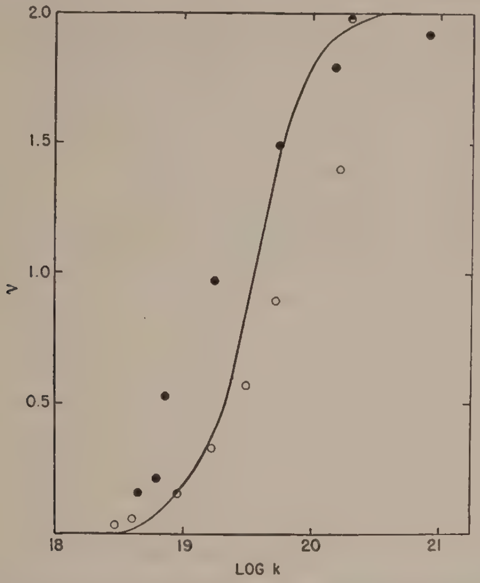

Fig. 2 Data of figure 1 corrected for proton affinity. The solid line is the theoretical curve calculated from the final values of the equilibrium constants. (Legend same as for fig. 1.)

Since the model chosen to make the correction for acid dissociation is an arbitrary one, the value obtained for pk_A may seem to be equally arbitrary. However, all of the models which showed any tendency to bring together the extrapolation points of the two curves did so in the region of $pk_A = 10$, so that there is reason to believe that this value is fairly independent of the model chosen.

The ionic strengths of the solutions of tables 1 and 3 differ somewhat. If we calculate the ionic strength of sea water from the average concentration of ions (Henderson, '13), we obtain a value of 0.67. It is known that *Limulus* serum possesses a salt concentration similar to that of sea water (Krogh, '39). This enables us to estimate the ionic strength of the solutions of tables 1 and 3. They are 0.08 and 0.11 to 0.12 respectively. It seemed possible that such a difference in ionic strength might account for the differences between the two curves of figure 1. A set of experiments was performed in which the ionic strength was raised to 0.22, and the pH varied (without buffer) by the addition of CN^-. The resulting points coincided exactly with the curve of table 1 where the pH was about 8.1, and with the curve of table 3 where the pH was about 8.6. This indicates that the pH, not the small variation in ionic strength, is the primary factor influencing the position of the curves.

TABLE 4

Limulus serum, equilibration in absence of oxygen
KCN concentration: 7.12×10^{-3} *M.*
Copper concentration in all final solutions: 0.388 mM.

KCN CC. ADDED	OXYHEMOCYANIN %	pH	ν	LOG k
2.00	63.9	7.37	1.28	17.70
3.00	45.4	7.58	0.907	17.79
4.00	35.1	7.78	0.701	17.73

Table 4 and figure 1 show the results of the experiments performed in vacuum, which were described at the beginning of this paper.[4] The uncorrected data are not at all in agreement with the other data of figure 1. When the points are recalculated with the inclusion of the acid constant, however, there is good agreement with the other experiments (fig. 2). The point obtained in vacuum shown in figure 2 represents that point with the lowest value of ν. The other points had such high corrected values of ν (greater than 1.99) that it was impossible to obtain an accurate value of k by calculation. The point given therefore represents a lower limit for the value of k given by the vacuum data alone.

[4] These experiments used 2 ml of serum, with water and KCN added to make a total volume of 12 ml. No buffer was used.

DISCUSSION

The validity of this analysis depends upon the reversibility of the dissociation of copper from the protein. Although no direct demonstration of complete reversibility has been obtained, several observations seem to indicate that the reaction is reversible. Pearson ('36) attempted to demonstrate the reversibility of the cyanide-hemocyanin reaction by treating the decolorized mixture with oxygen under pressure. His results were negative, for obvious reasons. He also allowed decolorized specimens to remain in contact with air in such a way that HCN could escape, and looked for an increase in the blue color. His results here, too, were negative. A repetition of the latter experiment shows that if the material is allowed to stand for a period of about two weeks, it will begin to regain its color. The work of Kubowitz ('38), in addition, demonstrates that it is possible to replace the copper on the protein and regain 50% of the physiological activity. Kubowitz was never able to regain all of the original activity, probably because of the rather harsh treatment accorded the protein in replacing the cuprous ion. Even if the other 50% of the protein had been inactivated at the binding sites by the large excesses of cyanide, the fact that 50% of the activity remains indicates that the value of k_1 could at worst be too small by a factor of two. This would increase log k_1 to 18.7, which in no way invalidates the conclusion drawn in this paper. The absence of abrupt changes in the direction of the curves suggests that there is actually very little inactivation during the removal of the copper.[5]

The very slow rate of recombination of protein with copper observed in the experiments with deoxygenated hemocyanin has made it impossible to reach the equilibrium position from both directions. It is consequently not possible to state with certainty that the equilibrium position has been reached in the experiments performed with oxygenated hemocyanin. Though the extinction coefficient of the protein solutions appeared to remain constant for some days, it is possible that a very slow dissociation reaction was occurring. The close agreement of dissociation constants calculated from experiments on both sides of the equilibrium position reassures us that the error is not a very large one.

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[5] Experiments of Lessiau, et al., on horse serum albumin ('48) suggest that possible irreversible effects of cyanide on the non-specific portions of the protein molecule, such as reduction of disulfide linkages, are of negligible importance at the cyanide and hydrogen ion concentrations used in removing the copper from hemocyanin.

In *Limulus* and *Busycon* sera at least 90% of the protein is hemocyanin. The other proteins present do not appear to carry any copper (Montgomery, '30). The presence of any impurity in the serum should, therefore, have very little effect upon the resultant constants.

The high value of the equilibrium constant for the addition of the first copper to each site is not similar to that of any simple cuprous complex, with the exception of cuprous-sulfhydryl complex (Bjerrum, '50). Stricks and Kolthoff ('51) have found a value of 1.5×10^{19} for the cuprous-cysteine association constant. Furthermore, the value of 10 for pk_A is consistent with sulfhydryl group behavior. A pair of amine groups would probably bind copper ion with a similar strength, however, so that the results do not indicate conclusively that a sulfhydryl group is involved. The possible existence of a prosthetic group which is composed of structures not directly part of amino acid side chains must not be overlooked.

The author gratefully acknowledges the advice given him by Dr. Alfred C. Redfield throughout the course of this work. He also is indebted to Dr. Margaret J. Hunter for her suggestions and assistance, and to Dr. Linus Pauling, Dr. Walter L. Hughes, Dr. Irving Klotz and Dr. Martin Karplus for helpful discussions. The author thanks the Woods Hole Oceanographic Institution for providing a fellowship, during the tenure of which this work was carried out.

SUMMARY

The binding of copper ion by *Limulus* and *Busycon* hemocyanins has been studied, by allowing cyanide ion to compete for the copper.

The pH-independent constant for the binding of the first copper ion to a site on *Limulus* hemocyanin has been found to be approximately $10^{18.7}$. The constant for the second copper ion is about $10^{20.5}$. *Busycon* hemocyanin has a first constant of approximately $10^{16.8}$, at a pH of about 8.1. In the case of *Busycon*, no attempt was made to determine a true, pH-independent, constant.

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ELECTRICAL AND CURVATURE RESPONSES OF THE AVENA COLEOPTILE TO UNILATERAL ULTRAVIOLET IRRADIATION[1]

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FIVE FIGURES

INTRODUCTION

Unilateral irradiation of the coleoptile of *Avena sativa* by moderate dosages induces bending toward the radiation source. Action spectra, made by irradiating only the apical segment or the entire seedling or by using a differential lighting method, show that this response increases as the wave lengths below 600 mμ become shorter (Blaauw, '14, '15, '19; Königsberger, '23). This reaction has a maximum at 436 mμ, decreases as the wave lengths get shorter (Sonne, '28, '29) and becomes insignificant below 300 mμ.

Phototropism depends upon an unequal distribution of auxin in the coleoptile (Went and Thimann, '37; Boysen-Jensen, '36). A recent review (Galston, '50) fails to identify the specific photoreceptor in the visible spectrum, nor has it been established how the unequal distribution of auxin is mediated (Thimann, '39). For wave lengths shorter than violet, the evidence indicates that auxin is inactivated by conversion to a lactone (Laibach and Maschmann, '33; Kögl, '36; Koningsberger, '36). This could result in an unequal distribution of active auxin in the unilaterally illuminated coleoptile.

It has been demonstrated that human beings (Saidman, '25), leaves (Sheard and Johnson, '29) and chicken eggs (Ro-

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[1] Aided by funds from the Atomic Energy Commission.

manoff, '43) manifest electrical responses to stimulation by ultraviolet light. The bioelectrical pattern of the Avena coleoptile is sensitive to visible radiant energy (Backus and Schrank, '51), but apparently no one has studied the electrical responses to ultraviolet irradiation.

Since the available ultraviolet data are rather incomplete, it seemed relevant to extend the investigation of the Avena curvature responses to irradiation of wave lengths of 260 to 400 mμ and concurrently observe any effects on the inherent electrical pattern.

MATERIALS AND METHODS

The test material was *Avena sativa* (Victory Strain C. I. 2020).[2] Seeds were husked, soaked, sprouted under weak red light on moist filter paper in petri dishes, transplanted to individual holders and grown in aquaria. All solutions were phosphate buffered tap water. Coleoptiles which had reached a length of 30 (± 2) mm were selected for straightness and general uniformity. Except for the Avena test, each experiment with intact plants was duplicated using isolated coleoptiles. Curvatures were measured by making shadowgraphs which were projected, traced and the amount of bending read from the tracing. Other measurements were made by following the horizontal tip movement with an ocular micrometer mounted in a low power microscope.

The electrical measuring instrument was a direct current amplifier (du Bridge and Brown, '33) with a Rubicon galvanometer (model 3403 HH) as the indicating device. Electrical contact to the coleoptile was made by a meniscus of Shive's solution in glass tubes 1 mm in diameter, connected to the amplifier by isoelectric Zn, $ZnSO_4$ electrodes. All experiments were performed in a dark room at a temperature of 24°C. (± 3) under a neon light in a ruby glass tube which emitted only wave lengths longer than 607.4 mμ.

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[2] Supplied by the U. S. Department of Agriculture.

The source of radiation was a Mazda ultraviolet AH-4 low pressure mercury arc with window cut in the outer glass envelope. The lamp was mounted in a transite-lined, light-tight box with an opening to accommodate the required filters. A cardboard shutter operated in front of the filter. The intensity of the filtered irradiation was measured with a Moll large surface thermopile which had been calibrated against a standard lamp. The energy delivered to the test material was obtained at a given distance from the lamp by multiplying ergs/cm^2 by the exposure time. During exposure black paper was placed on two sides of the coleoptile to shield it from reflections.

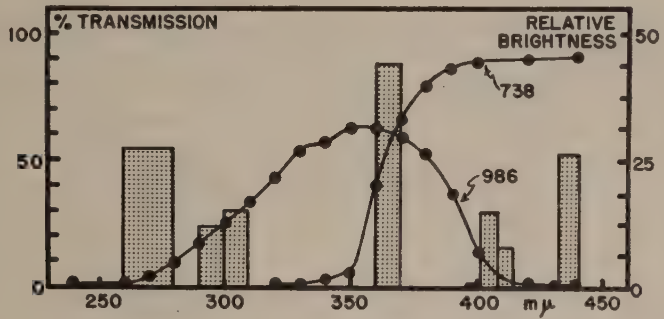

Fig. 1 Filter transmission curves. Ordinate on the left indicates per cent transmission. The abscissa is wave length in mμ. The vertical bars represent the various mercury lines at the given wave length. The ordinate on the right gives the relative brightness of the mercury lines.[3] The curves are numbered with Corning filter numbers.

EXPERIMENTAL RESULTS

Figure 1 shows the transmission curves of the filters measured with a Beckman model DU quartz spectrophotometer. The bars representing relative brightness are based on data furnished by the General Electric Company.[3] When filter number 986 was used all the Hg lines between 265 mμ and 435 mμ were transmitted while filter number 738 transmitted wave lengths longer than 350 mμ.

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[3] The data for the relative brightness shown in figure 1 were kindly supplied by Dr. L. J. Buttolph of The General Electric Company.

Curve I, figure 2 A, represents final curvatures of the intact plants, which were allowed variable times to reach maximum curvature. The time required for each dosage was determined by preliminary experiments. At each dosage there were two experimental conditions. In one case the bending occurred under the neon light and in the other case in a dark box. The maximum difference between final curvatures in the red light and the dark occurred when $6.96 \times (10^5$ ergs/cm^2)[4] were applied. Plants responding in red light had an average curvature of 15 degrees with an individual variation of 8 to 23 degrees while coleoptiles responding in the dark had an average curvature of 11 degrees and a variation of 4 to 18 degrees. Since the "t" test indicates that the two mean curvatures are not significantly different ($p>0.5$) an average of

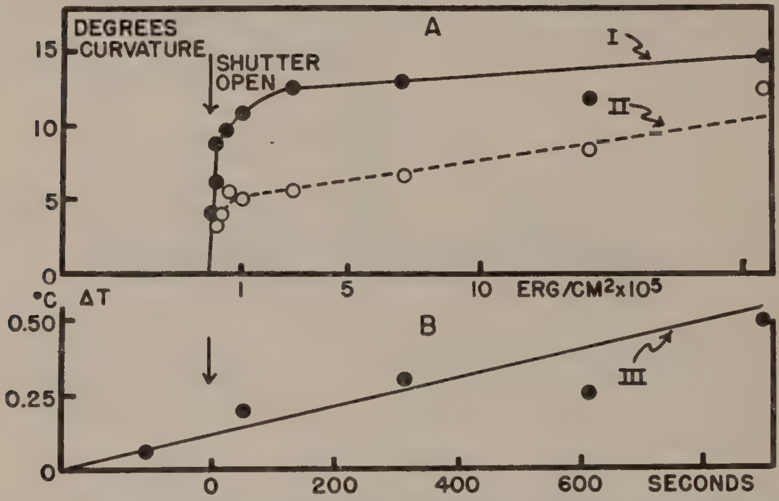

Fig. 2 A Final average curvatures of Avena coleoptiles in response to unilateral irradiation with various dosages of ultraviolet light. Curve I is for intact plants; curve II is for isolated sheaths.

Fig. 2 B Temperature changes.

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[4] Throughout this paper the factor 10^5 ergs/cm^2 is represented by the letter d.

40 individuals was taken for each point in curve I, half responded in red light and half responded in the dark. This curve shows a steeply increasing curvature response with increases in dosage up to 0.35d and then a smaller increase in response with each increase in dosage. Only slight increases in curvature are apparent with increases in irradiation up to 83.5d.

Curve II, figure 2 A, is from a duplicate set of experiments performed on isolated coleoptiles. Similar results were obtained except that final curvatures were always less for a given dosage. The average maximum at 20.9d is 15 degrees for the intact plants and 11 degrees for the isolated sheaths. As in the intact plant, the differences between the final curvature in the red light and in the dark are not statistically significant.

At 1.39d the average response of intact plants is 11 degrees whether the reaction occurs in the dark or in the red light. Nineteen individuals were exposed to this dosage with the transport bundle closest to the lamp. These plants produced curvatures varying from 3 to 16 degrees with a mean of 12. The other experiment included 27 individuals which were exposed with the longest transverse axis at right angles to the light beam. Their variation was 4 to 19 with a mean curvature of 10 degrees. The two mean curvatures are not statistically different, thus the magnitude of bending is independent of the side of the coleoptile irradiated.

Maximum curvature responses to unilateral irradiation of 18.6d were determined. Plants were unilaterally exposed by all of the Hg lines from 265 mμ to 435 mμ. The final average curvature was 13 degrees. Coleoptiles exposed to the same dosage but only to wave lengths of 350 mμ to 435 mμ had an average curvature of 2 degrees. Therefore only 16% of the total curvature is induced by wave lengths longer than 350 mμ.

Curve III, figure 2 B, is a record of temperature changes occurring during the time that the lamp was on. The lamp was activated at least 4 minutes before the shutter was opened to establish a given standard output. To keep the temperature increase at a minimum, especially during the long exposures, a stream of air was forced over the box. A thermometer was placed a little to one side and 2 cm closer to the lamp than the coleoptile, so that the mercury bulb received a full intensity of the irradiation. These temperatures were recorded while observing tip movement and electrical changes. The points of curve III represent average temperature increases at a given time after the lamp was activated. In only one case was there a temperature decrease while the lamp was on. In 35% of the experiments no temperature change was indicated while the shutter was closed. The points do not fall precisely on a straight line because of the intermittent operation of the temperature regulating mechanism in the laboratory. Individual experiments at different dosages show no relationship between temperature increase and curvature. For example at 6.96d with a temperature increase of 0.6°C. some coleoptiles bent 10 degrees, while others bent 20 degrees. In one case the temperature decreased 0.1°C. and the coleoptile developed a curvature of 14 degrees. Similar assortments of temperature changes and curvatures occurred throughout the range of dosages used.

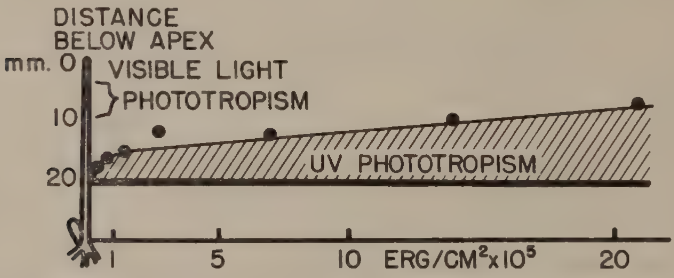

Fig. 3 Changes in the region of response of intact coleoptiles with changes in dosage of irradiation.

Figure 3 shows which region is involved in the curvature. Data were obtained by measuring from the apex to the beginning of the curvature on the shadowgraphs of the intact plants of curve I, figure 2 A. At the lowest dosage used, 0.116d,

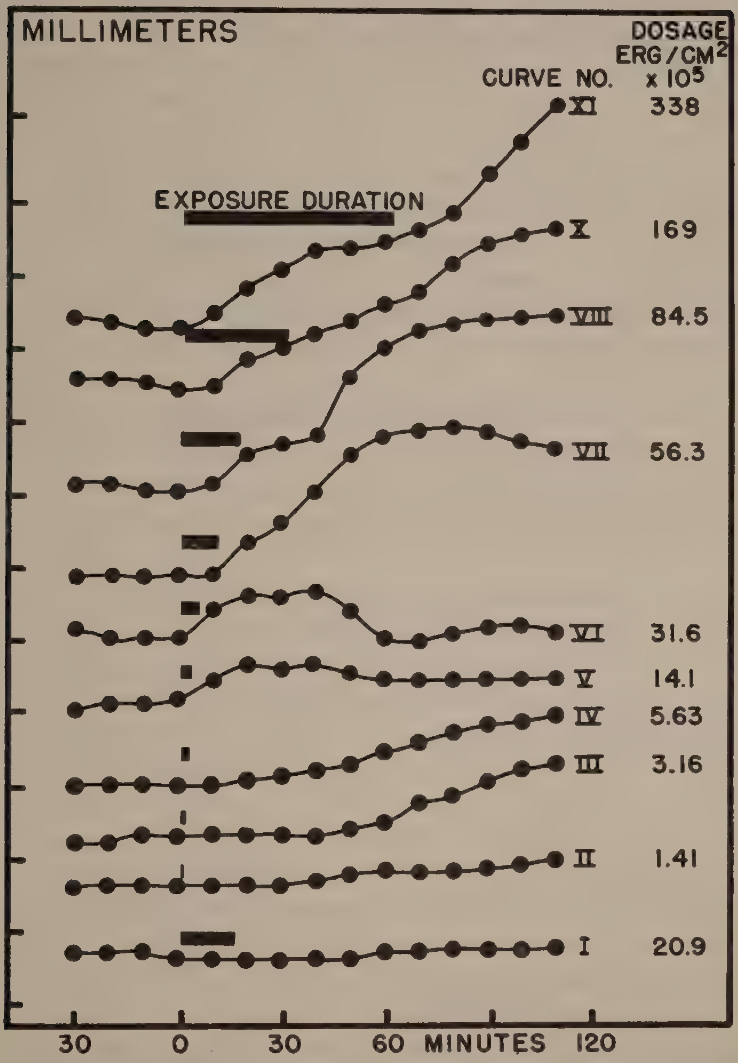

Fig. 4 Average horizontal tip movement of isolated coleoptiles. Each division on the ordinate represents 2 mm. An upward excursion of the curve indicates movement of the apex toward the source of irradiation. The curves are vertically displaced for ease in reading. The Arabic numeral at the right shows the dosage in ergs/cm² × 10⁵. The solid bar above each curve indicates the duration of irradiation.

the first noticeable bending occurred 19 mm below the apex. As the dosage is increased the region of the first bend moves upward. These shadowgraphs also show that additional increments of the coleoptile are affected as the dosage is increased.

Ultraviolet induced curvature is somewhat different than the curvature resulting from visible light. With visible light the curvature is ''angle-like'' and occurs 5 to 10 mm below the apex of the plant. As the dosage of visible light is increased the angle of the bend becomes sharper. Ultraviolet irradiation produces a curvature that is ''arc-like,'' bending first near the base. As the dosage of ultraviolet is increased more tissue responds producing greater curvature. The appearance of the curvature at low dosages of ultraviolet light, 0.116 to 0.348d, is ''angle-like'' as the phototrophic response except that the bend occurs about 20 mm, rather than 5 to 10 mm below the apex.

Figure 4 shows the horizontal tip movement of isolated coleoptiles. Curves I through V are averages of 5 individuals while curves VI through XI are averages of 10 individuals. The curvature responses to ultraviolet light have two common characteristics. First the coleoptile always bends toward the light regardless of the dosage. Second there is an increase in magnitude of curvature as the dosage is increased. Information about the mechanisms involved in the curvature responses is also revealed. Curve I represents the bending response of seedlings that were decapitated twice as in the Avena test. These plants, which were without natural auxin, did not bend. Plants which had bent were inspected with a low power microscope, and no evidence of injury or loss of turgor was revealed. These observations are interpreted to mean that the bending is accomplished by auxin requiring growth processes.

Three kinds of curvature responses are observed when tip movement is studied. Below 5.63d there is a bending toward the light that starts about 30 minutes after the beginning of exposure and reaches the maximum 60 to 90 minutes later.

The initial curvature stops and the coleoptiles bend away from the light. The second and third phases are not shown on the graph. From 14.0d to 31.6d bending toward the light starts 4 to 10 minutes after the shutter was opened and lasts for 20 to 30 minutes. This is followed by a decrease in the rate of bending toward the lamp, and finally a bending away from the lamp. When the tip returned to 0, as indicated by curve VI, the coleoptile is "S" shaped rather than straight. Such return to 0 occurred in plants that were irradiated with less than 31.6d. At 84.5d the curve levels off 90 minutes after the beginning of exposure but does not return to 0. The third type of response is represented by curves X and XI. These coleoptiles show tip movement toward the lamp 4 to 10 minutes after the beginning of exposure and continue in this direction. Data not on the graph show that bending toward the light source at 169d continues for 150 minutes and at 338d the bending in the initial direction is maintained for 180 minutes. These experiments were too short to see a return toward 0. These data show that the time required to reach the maximum curvature in the initial direction increases as the irradiation is increased from 31.6d to 338d.

At 14.0d the average maximum curvature of intact coleoptiles is 10 degrees, while isolated sheaths have an average maximum curvature of 9 degrees.

At this point it became important to determine the effect of ultraviolet irradiation on indole-3-acetic acid. A water solution of 0.4 mg/l was prepared and aliquots were used in the different experiments. The IAA was applied unilaterally in agar blocks 1.8 × 2 × 2 mm to the transport bundle side of the coleoptile. A response time of 100 minutes was allowed. To eliminate hourly and daily variations the experiment consisted of three parts preformed simultaneously. Coleoptiles were selected and used in the Avena test as follows. In the control group neither the coleoptile nor the IAA was irradiated. The agar block containing IAA was unilaterally placed on the coleoptile and the curvature determined. In the second group the entire coleoptile was irradiated with 31.6d using filter number 986. After irradiation a block of agar containing IAA was placed on the coleoptile. In the third group agar blocks containing IAA were irradiated with 31.6d and then applied to the prepared coleoptiles. Results of a typical experiment (A) and a summary (B) are given in table 1. The region of the bend in the controls and the plant treated with irradiated IAA is 5 to 10 mm below the apex and the curve is "angle-like." With irradiated coleoptiles the bend occurs 10 to 15 mm below the apex and the curve is "arc-like."

TABLE 1

Changes in IAA activity and plant sensitivity produced by ultraviolet irradiation

	CONTROL	IRRADIATED IAA	IRRADIATED COLEOPTILE	EXPERIMENT NUMBER
	19	5	8	I
A	5	3	3	
	10	6	13	
Degrees of	9	7	7	
curvature	12	5	5	
	11	5	7	Average
	0	53	35	I
B	0	50	39	II
Per cent	0	63	30	III
curvature	0	61	39	IV
decrease	0	55	31	V
	0	56	35	Average

In the final group of experiments the electrical responses to ultraviolet light were observed. After the coleoptiles were placed in individual holders, the contacts were adjusted and the recording begun. All experiments were started 30 minutes before the shutter was opened to establish the nature of

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Fig. 5 Longitudinal electrical polarity changes of individual isolated coleoptiles. Each division of the ordinate represents 20 mv. A downward excursion of the curve indicates an increase in potential difference. The magnitude of the initial electrical polarity of each coleoptile is shown by the Arabic numeral and dash at the left of the curve. The Arabic numeral at the right shows the dosage of irradiation in ergs/cm^2 × 10^5. Duration of irradiation is shown by the solid bar above each curve. The curves are vertically displaced for ease in reading.

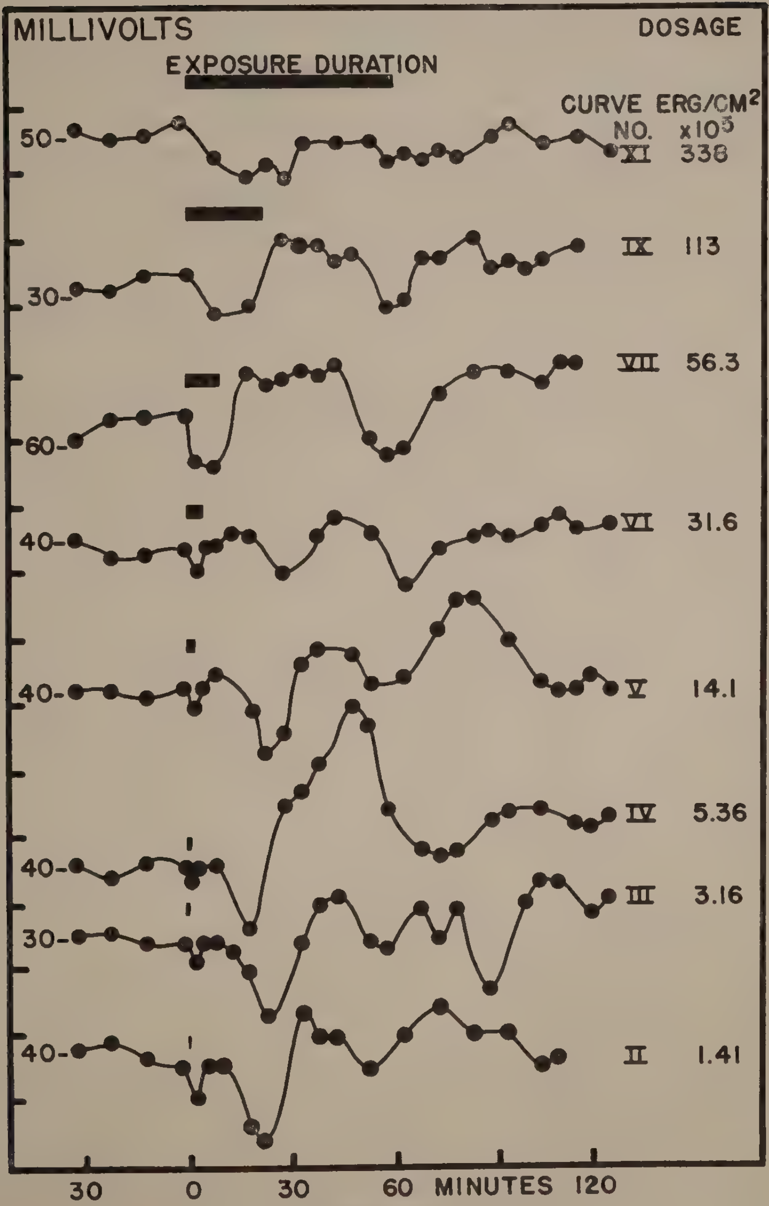
MILLIVOLTS
DOSAGE
EXPOSURE DURATION
CURVE NO.
ERG/CM² x10⁵
XI 338
IX 113
VII 56.3
VI 31.6
V 14.1
IV 5.36
III 3.16
II 1.41
50-
30-
60-
40-
40-
40-
30-
40-
30
0
30
60
MINUTES
120

Figure 5

of the inherent potential of each individual. Either the basal or the shaded side contact was grounded and the other contact was connected to the control grid. Various contact positions were used, beginning with the region 1 mm from the apex and then every successive 5 mm below the apex. The transverse potential was measured across the short axis of the coleoptile and for the longitudinal measurements the contacts were placed on the flat side. The maximum responses were found when the apical contact was placed 5 mm below the apex and the basal contact was placed 25 and 30 mm below the apex on isolated sheaths and intact coleoptiles, respectively.

Figure 5 shows the longitudinal potential changes of the lighted side of isolated coleoptile sheaths. Each curve was selected because it most nearly matched the average of 5 or more similar curves. A downward excursion of the curve indicates an increased potential difference. The initial longitudinal potential varied from 30 to 60 mv., with the apical region negative to the basal portion.

Characteristics common to all dosages are one or more transitory increases in longitudinal polarity. A general increase in magnitude and number of waves of electrical polarity change occurs up to 5.63d after which there is a general decrease in the magnitude, decrease in the number of phases and lastly a decrease in the variability. After the transitory increases in potential, there is a return to the prestimulation level of magnitude and variability. Another common point is a latent period or delay between turning the lamp on and the changes in the potential. This period is too short to be evident in the graphs.

The curves are divided into two groups on the basis of the response patterns. One group received dosages less than 31.6d (curve numbers II through VI) while the second group received larger doses (curves VII through XI). At the lower dosages there are three transitory increases in potential and the responses are faster and more complex than at higher dosages. Curve IV illustrates the maximum electrical responses observed in the lower range. The magnitude of the responses increase up to 5.63d and then decrease throughout the remainder of the dosage range. These curves show a small rapid increase in potential that begins within 60 seconds after the exposure, reaches its maximum in two minutes, and returns to the original level in 4 minutes. This decrease in polarity does not depend on turning off the lamp as can be seen from the exposure duration bar above the curve. Similar changes in electrical polarity occur in 95% of the plants, a few of which show the response only on the lighted or the shaded side.

The second transitory increase in potential in response to low dosage irradiation is longer in duration, greater in magnitude and slower to appear than the first. This change begins about 12 minutes after irradiation, reaches its maximum in about 22 minutes and returns to the original level in about 30 minutes. The subsequent decrease in longitudinal potential occurs most noticeably at lower dosages. The last electrical response to low dosage is a third wave of increased potential difference. Its maximum appears about 50 minutes after stimulation and has a duration of about 20 minutes after which it returns to the original base level.

In the lower dosage range the lighted side responds faster than the shaded side and the magnitude of its change is slightly smaller. These differences are most evident during the first phase of the response.

When the contacts were placed 5 mm below the apex and on opposite sides of the coleoptile the maximum transverse polarity was observed. The potential changes, primarily during the first phase of the longitudinal response pattern, are such that the lighted side becomes electronegative to the shaded side. The transverse electrical polarities observed during other phases of the longitudinal response at other levels below the apex and at other dosages tested showed no significant changes.

The electrical responses to the higher dosages are illustrated best by curve VII. Only two transitory increases in

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longitudinal potential are evident. There is an absence of the first, or a fusing of the first and second phase, and an absence of the decreased polarity that followed the second transitory rise. The disappearance of this phase first occurred above 14.1d. Finally a noticeable decrease in the variability of the electrical polarity is apparent.

DISCUSSION

Unilateral irradiation by ultraviolet light causes the Avena coleoptile to bend toward the source of irradiation and causes changes in some parts of its inherent electrical field. In the present study the most effective wave lengths were shorter than 350 mμ, the range in which Sonne ('28–'29) found practically no effect. In determining the action spectrum Sonne irradiated the auxin synthesizing centers with just enough monochromatic light to produce incipient bending. In contrast, in the present experiments the entire coleoptile was unilaterally irradiated. The difference in sensitivity at the different regions of the spectrum may be due to this difference in experimental procedure. The first noticeable bending occurred in cells that are most sensitive to the depletion of auxin (Thimann, '34). As the dosage is increased, additional cells that are less sensitive to auxin depletion respond, producing an "arc-like" bend, typical for ultraviolet irradiated plants. It was demonstrated that these "arc-like" curvatures are distinctly different from phototropic curvatures produced by visible light.

Laibach and Maschmann ('33), Kögl ('36), and Koningsberger ('36) maintain that ultraviolet light destroys the activity of auxin a. The present experiments show that IAA is also partially inactivated by ultraviolet irradiation. However the bending response cannot be simply the depletion of auxin because, when decapitated coleoptiles are irradiated and then subjected to IAA, there is a decrease in the magnitude of curvature and it is of the "arc-type." Thus there must be two different effects of ultraviolet irradiation upon the auxin requiring growth processes; one, the destruction of auxin activity and the other, some effect on the cells that persists for some time after irradiation. The nature of the latter effect has not been established and its clarification requires additional experiments.

Ultraviolet irradiation also causes some changes in the inherent electrical field of the coleoptile. It has been previously demonstrated (Backus and Schrank, '51) that unilateral irradiation by visible light causes the establishment of a transverse electrical polarity. Experiments were carried out to determine whether such polarities are established in response to ultraviolet irradiation. No significant transverse polarities were observed at any level of the coleoptile. This apparently means that the mechanism involved in the "persisting effect" induced by ultraviolet irradiation is different from the process initiated by unilaterally applied visible light. The longitudinal component of the electrical field does undergo a series of changes. Previous experiments have shown that the depletion of auxin in the coleoptile has no effect on the longitudinal potentials (Mills and Schrank, '50). For this reason these electrical polarity changes apparently are not caused by the photodestruction of auxin. However it remains to be established whether the longitudinal electrical changes induced by ultraviolet irradiation are in some way related to the "persisting effect" that induces the "arc-like" curvature.

SUMMARY

1. Unilateral irradiation with wave lengths between 250 and 400 mμ causes the Avena coleoptile to bend toward the radiation source. The most effective wave lengths were shorter than 350 mμ. Increases in dosages of irradiation up to 20×10^5 ergs/cm^2 cause increases in the magnitude of curvature.

2. The evidence presented indicates that the bending is due to growth curvatures which require auxin.

3. At least two mechanisms are involved in the induction of these curvatures. Firstly, irradiation inactivates indole-3-acetic acid. Secondly, irradiation produces a change in the

cells that persists after irradiation and causes an alteration in the action of auxin. The nature of this second effect has not been clarified.

4. Curvatures induced by unilateral ultraviolet irradiation are qualitatively different from phototropic curvatures as indicated by the differences in the regions of the coleoptiles that produce the bending.

5. Various electrical responses to ultraviolet irradiation were also studied. No significant changes were observed in the transverse electrical polarity, but the longitudinal component of the inherent electrical pattern on the lighted as well as the shaded side is sensitive to ultraviolet irradiation. These polarity changes are transitory wave-like increases in potential difference whose complexity and magnitude are related to the dosage of irradiation.

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THE INTRACELLULAR DISTRIBUTION OF CYTOCHROME COMPONENTS AND OF OXIDATIVE ENZYME ACTIVITY IN RAT LIVER[1]

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FOUR FIGURES

It is well-known that the major pathway of biological oxidations in aerobic organisms involves the transport of electrons from dehydrogenating enzymes to oxygen through a chain of cytochrome pigments, including cytochromes a, b, c and cytochrome oxidase. These pigments are readily identified by their characteristic and intense absorption spectra in the reduced state, and have been observed in most actively metabolizing tissues by spectroscopic examination of thin sections, suspensions or homogenates of these tissues. It is therefore of interest that the cytochrome spectrum of liver, as well as of certain other visceral organs, is quite different from that observed, for example, in heart muscle. When a suspension of liver cells is reduced with dithionite (sodium hydrosulfite) and examined with a direct vision prism spectroscope, no distinct absorption bands of the "normal" cytochrome components are observed; instead an intense broad band is seen in the region 550–565 mμ. Nevertheless, enzymatic assays of liver fractions obtained by differential centrifugation have shown that the liver cells and in particular the mitochondrial fraction possess a high content of various oxidative enzyme

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systems, including succinate oxidase (Schneider and Hogeboom, '50), choline oxidase (Kensler and Langeman, '51), oxalacetate oxidase (Schneider and Potter, '49), cytochrome c reductases (Hogeboom, '49; Hogeboom and Schneider, '50a) and cytochrome oxidase (Schneider, '46). Furthermore, the isolation of cytochrome c from liver fractions has been reported, and a large percentage of the pigment recovered appears to be associated with the mitochondrial fraction (Schneider and Hogeboom, '50).

In the present study the cytochrome system of mammalian liver has been re-investigated, using whole liver homogenates and liver cell fractions obtained by a standard procedure for differential centrifugation. Analyses of the fractions in terms of dry weight, protein and lipid content are reported along with studies on the distribution of certain pertinent oxidative enzyme activities in these fractions. Previously reported studies of this nature deal largely with either one or the other of these aspects but not both. Also, an integration and comparison of previous results is made uncertain because a variety of suspending media and centrifugal forces were employed for the fractionation procedures in the various investigations. Finally, spectroscopic and spectrophotometric observations on the distribution of cytochrome pigment in the liver homogenate and fractions are reported and are correlated with oxidative enzyme activities.

EXPERIMENTAL

Liver fractionation

Preparation of liver homogenates. Adult male rats, weighing about 250 gm, were sacrificed by decapitation and their livers were thoroughly perfused *in situ* with ice-cold isotonic sodium chloride via the hepatic vein until the organ was blanched and gross signs of blood had disappeared. Thorough perfusion is imperative if reliable spectroscopic observations are to be made on the intrinsic hemochromogen components of liver cell fractions. The liver was then excised, rinsed with ice-cold water, blotted and the desired amount of tissue weighed out. The part of the liver attached to the diaphragm was not used since this region contains large blood vessels, much connective tissue and other non-parenchymatous cells.

The subsequent homogenization and fractionation were carried out at 0–5°C. with ice-cold media. The liver tissue was first minced with scissors and then thoroughly homogenized in two volumes of 0.25 M (isotonic) sucrose in an all-glass Potter-Elvehjem type homogenizer. Then two volumes of isotonic sucrose were added to make the final homogenate (Preparation H). This thick, opaque suspension was tan in color, with only a slight pink tint, and appeared to be grossly free of hemoglobin.

Fractionation of homogenates by differential centrifugation. Aliquots (usually 40 ml) of the liver homogenate (Preparation H) were fractionated by the procedure outlined in figure 1. The entire fractionation was completed within 5 hours from the time the rat was sacrificed, and the fractions were stored at 0–5°C. until used, generally the same day.

The nuclear fraction (N) as centrifuged out was composed of a whitish bottom layer of connective tissue fragments and unbroken cells, covered by a bulky, tan-white layer which microscopically was observed to consist mainly of nuclei with some admixture of mitochondria. Usually a minute deposit of red blood cells was found near the junction of the two layers. The mitochondrial or large granule fraction (L) was obtained in the form of opaque, tan-colored pellets. When stained with 1:10,000 Janus green B, which is specific for the large granules, this fraction was observed microscopically to be composed mostly of mitochondria. When first sedimented out, the submicroscopic particle or microsome fraction (M) was obtained in the form of completely transparent dark amber, jelly-like pellets. If the liver was not first perfused, the microsomes were contaminated with adsorbed hemoglobin, and consequently possessed a striking cherry-red color to which allusions were made in the early literature

(Bensley, '48; Claude, '41). The supernatant fraction (S) was a yellowish, slightly turbid liquid. The turbidity is due to components which can be separated out as a white scum-like surface layer at high centrifugal forces, i.e., 100,000 g (times the force of gravity) for 15 minutes.

Chemical analysis of fractions. For these determinations the liver fraction in aqueous suspension or the dilute liver homogenate was treated with 10% trichloroacetic acid. The precipitated material was collected by centrifugation, then

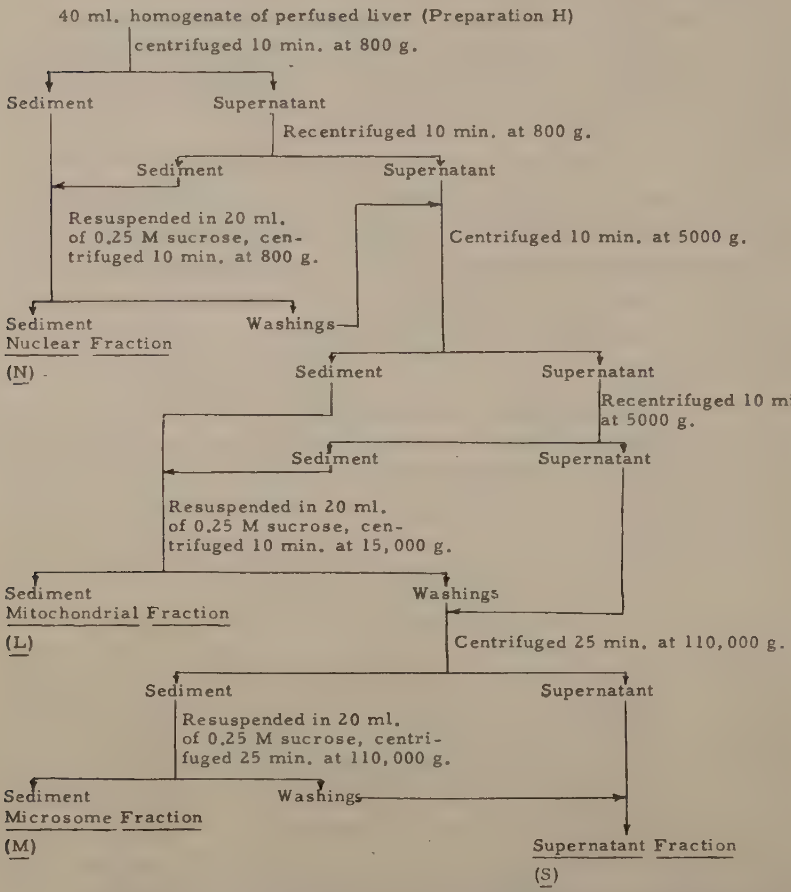
40 ml. homogenate of perfused liver (Preparation H)
centrifuged 10 min. at 800 g.
Sediment
Supernatant
Recentrifuged 10 min. at 800 g.
Sediment
Supernatant
Resuspended in 20 ml. of 0.25 M sucrose, centrifuged 10 min. at 800 g.
Centrifuged 10 min. at 5000 g.
Sediment
Nuclear Fraction
(N)
Washings
Sediment
Supernatant
Recentrifuged 10 min. at 5000 g.
Sediment
Supernatant
Resuspended in 20 ml. of 0.25 M sucrose, centrifuged 10 min. at 15,000 g.
Sediment
Mitochondrial Fraction
(L)
Washings
Centrifuged 25 min. at 110,000 g.
Sediment
Supernatant
Resuspended in 20 ml. of 0.25 M sucrose, centrifuged 25 min. at 110,000 g.
Sediment
Microsome Fraction
(M)
Washings
Supernatant Fraction
(S)

Fig. 1 Scheme for fractionation of liver homogenates by differential centrifugation.

washed once with 50% ethanol and once with 95% ethanol. The resulting residue was used for the analyses. For "dry weight" determination the residue was dried by heating in an oven at 110°C. for two hours, stored overnight in vacuo over calcium chloride, and then weighed. For "protein" determination the residue was extracted three times for 10 minutes with 25 volumes of boiling 2:1 absolute ethanol: ether, and the residual material was dried as in the dry weight analyses and weighed. The ethanol: ether extracts were pooled, taken to dryness over a steam bath and weighed to obtain the "lipid" content.

TABLE 1

Composition of rat liver fractions

FRACTION	DRY WEIGHT (minus TCA-soluble material)		PROTEIN (plus nucleoprotein)			LIPID (lipid associated with TCA precipitate; mostly essential lipid)		
	mg/gm of fresh liver	% of total dry weight	mg/gm of fresh liver	% of total protein	% protein in fraction	mg/gm of fresh liver	% of total lipid	% lipid in fraction
Homogenate (H)	193.2	(100)	149.2	(100)	77.3	39.3	(100)	20.3
Nuclei + "Debris" (N)	35.3	18.3	30.2	20.3	85.7	4.0	10.2	11.3
Mitochondria (L)	47.0	24.3	34.1	22.8	72.6	12.8	32.6	27.3
Microsomes (M)	46.6	24.1	29.2	19.7	62.7	17.5	44.5	37.6
Supernatant (S)	62.4	32.3	54.5	36.5	87.5	6.0	15.2	9.6
Sum of fractions	191.3	99.0	148.0	99.3	...	40.3	102.3	

The results of such analyses on the liver homogenate and fractions are collected in table 1, with each value representing the average of at least 6 determinations. These data appear to be in essential agreement with such previously reported results as are comparable. Thus the distribution of protein among the fractions is similar to the distribution of total nitrogen calculated from the data in the literature (Ludwig and Chanutin, '50; Schneider and Hogeboom, '50). No comparable breakdown of lipid distribution in rat liver has been published, but scattered data are available for certain fractions. According to Swanson and Artom ('50) the lipid content of rat liver mitochondria is 28.5% of the dry weight.

The microsome fraction obtained from rat liver homogenate prepared in isotonic saline was stated by Claude ('46) to contain as high as 40 to 45% lipid.

Enzymatic composition of liver fractions

Assays were run on the liver homogenate and fractions for the following enzymes and enzyme systems: succinate oxidase, cytochrome oxidase, succinate dehydrogenase, and DPN-cytochrome c reductase. The assay methods employed involve both manometric and spectrophotometric techniques and in general follow standard procedures described in the literature. Each datum presented here is the average of values from at least 6 determinations. The liver preparations were dispersed to suitable dilution with ice-cold isotonic sucrose immediately before the tests.

Manometric determination of succinate oxidase. For all of the manometric assays to be described, the conventional Warburg procedure was followed, using an air atmosphere at 37.5°C. All of the vessels contained phosphate buffer, pH 7.4, in a final concentration of 3×10^{-2} M and 10% potassium hydroxide was used in the center wells. Except where otherwise noted, cytochrome c was added to each flask to give a final concentration of 10^{-5} M. In the succinate oxidase assay, sodium succinate was employed as a substrate in a final concentration of 1.3×10^{-2} M. The enzyme suspensions were added to the flasks last, the vessels were equilibrated for 7 minutes and the substrate was then tipped in from the side arm. Readings of oxygen uptake were begun after three minutes more for re-equilibration and were continued at 5- or 10-minute intervals. The oxygen consumption for the initial 20 minutes was used in subsequent calculations since during this period the uptake was linear. Blank readings, obtained with vessels containing no substrate, were subtracted from total oxygen consumption before calculating the enzyme activities.

The results of the succinate oxidase assays are collected in table 2. The differences in activities observed with and without cytochrome c indicate that in preparation of the liver homogenate and fractions the endogenous cytochrome is diluted out or displaced from its functional spatial orientation in the cell so that it becomes the limiting factor in the succinate oxidase system. It is clear from the values in the last column that the enzyme system is 3.0 times as concentrated in the mitochondrial fraction as in the original homogenate; in all of the other fractions the concentrations are considerably lower than in the homogenate. These data on the intracellular distribution of succinate oxidase activity are in good agreement with the findings of Schneider and Hogeboom ('50).

TABLE 2

The distribution of succinate oxidase activity in rat liver fractions

FRACTION	NO CYTOCHROME c ADDED	WITH ADDED CYTOCHROME c		
	Q_{O_2} — L [1]	Q_{O_2} — L [1]	% of total activity	Q_{O_2} — P [2]
H	6,640	13,600	(100)	91.2
N	650	1,440	10.5	48.2
L	4,810	9,460	69.6	276.8
M	280	480	3.5	16.4
S	50	200	1.5	3.6
Recovery	..	...	85.1	..

[1] Q_{O_2} — L — Oxygen uptake in microliters per hour per gram of fresh liver.

[2] Q_{O_2} — P — Oxygen uptake in microliters per hour per milligram protein in the liver fraction.

Manometric activity of cytochrome oxidase. The assay procedure for cytochrome oxidase activity was exactly like that for succinate oxidase except that 2×10^{-2} M p-phenylenediamine was used as substrate in place of succinate. Cytochrome c was added in a final concentration of 10^{-5} M.

In table 3 are collected the results of the cytochrome oxidase assays. The distribution of this enzyme reflects that of the complete succinate oxidase system: the major portion of the activity is recovered in the mitochondria, and again the mitochondrial fraction is the only point of enzyme concentration. No cytochrome oxidase assays on rat liver preparations in isotonic sucrose have appeared in the literature, but the values obtained on fractions from an alkaline-water homogenate (Schneider, '46) are in fair agreement with those reported here.

Manometric determination of succinate dehydrogenase. In this assay the cytochrome oxidase system was inhibited by cyanide, and methylene blue was used in place of the cytochrome system to carry electrons from succinate dehydrogenase to oxygen. Since cytochrome c is not involved in this artificial electron transport system, no excess cytochrome c was added to the Warburg vessels. Each flask contained 3×10^{-2} M phosphate, pH 7.4, 2×10^{-3} M cyanide and 10^{-3} M methylene blue; the substrate again was 1.3×10^{-2} M succinate. No alkali was added to the center wells; preliminary tests with whole homogenates proved its use to be unnecessary.

TABLE 3

The distribution of cytochrome oxidase activity in rat liver fractions

FRACTION	Q_{O_2} — L[1]	% OF TOTAL ACTIVITY	Q_{O_2} — P[2]
H	23,700	(100)	158.7
N	2,490	10.5	82.4
L	18,100	76.3	530.5
M	1,400	5.9	47.9
S	470	2.0	8.6
Recovery	...	94.7	..

[1] Q_{O_2} — L — Oxygen uptake in microliters per hour per gram of fresh liver.

[2] Q_{O_2} — P — Oxygen uptake in microliters per hour per milligram protein in the liver fraction.

The results of the succinate dehydrogenase assays are collected in table 4. The distribution pattern among the liver fractions is seen to be similar to that of the whole succinate oxidase system. These results are not surprising since this enzyme is usually the limiting factor in a succinate oxidase preparation to which an excess of cytochrome c has been added. It will be noted that the level of total oxygen uptake reported here is below that found in the succinate oxidase assay. This observation is not entirely unexpected in view of the artificial electron transport system employed here; methylene blue is not as effective a mediator of electron transfer as the natural mediator cytochrome oxidase (Potter, '41) and there may also be other limiting factors operative in the test system. No strictly comparable data can be culled from the literature, since succinate oxidase activity was taken as a measure of succinate dehydrogenase content in all of the distribution studies reported to date.

TABLE 4

The distribution of succinate dehydrogenase activity in rat liver fractions

FRACTION	Q_{O_2} — L[1]	% OF TOTAL ACTIVITY	Q_{O_2} — P[2]
H	5,650	(100)	37.9
N	570	10.1	18.9
L	4,010	70.1	117.6
M	430	7.6	14.7
S	110	1.9	2.0
Recovery	...	90.6	

[1] Q_{O_2} — L — Oxygen uptake in microliters per hour per gram of fresh liver.

[2] Q_{O_2} — P — Oxygen uptake in microliters per hour per milligram protein in the liver fraction.

Spectrophotometric determination of diphosphopyridine nucleotide (DPN) — cytochrome c reductase. The assay adopted for this determination is based on spectrophotometric observation of the rate of reduction of cytochrome c by reduced diphosphopyridine nucleotide (DPN·H). The reaction mixtures were made up by combining in order: 1.0 ml of 0.1 M phosphate buffer, pH 7.4; water to give a final volume of 3.0 ml; 0.1 ml of 0.8 M nicotinamide; 0.1 ml of 10^{-2} M potassium cyanide; 0.05 to 0.5 ml of a suitably diluted enzyme preparation; 0.3 ml of 3×10^{-4} M oxidized cytochrome c; and 0.2 ml of 2×10^{-3} M DPN·H. Control tubes contained

DPN·H but no enzyme. Immediately after addition of DPN·H, the mixtures were transferred to cuvettes and the increase in optical density at 550 mμ was followed with a Beckman model DU spectrophotometer at 23°C. The amount of enzyme preparation added in each case was sufficient to effect the reduction of half of the cytochrome c present in three to 10 minutes, and the optical density change for the first three minutes of readings was employed to calculate enzyme activity. In these calculations, values for the molar extinction coefficients of 9.0×10^3 cm^2 per mole and 2.81×10^4 cm^2 per mole (Umbreit, Burris and Stauffer, '45) were used for oxidized and reduced cytochrome c, respectively. The reduced diphosphospyridine nucleotide solutions used in the assay were prepared by the method of Hogeboom and Schneider ('50b) from a sample of 75% pure cozymase obtained from the Schwarz Laboratories.

TABLE 5

The distribution of DPN-cytochrome c reductase activity in rat liver fractions

FRACTION	ACTIVITY [1]	% OF TOTAL ACTIVITY	CONCENTRATION [2]
H	2,805	(100)	18.8
N	338	12.0	11.2
L	650	23.1	19.1
M	1,665	59.1	57.1
S	93	3.2	1.7
Recovery	..	97.4	

[1] Micromoles of cytochrome c reduced per hour per gram of fresh liver.

[2] Micromoles of cytochrome c reduced per hour per milligram protein in the liver fraction.

Results of these assays are indicated in table 5. The most striking point is that almost 60% of the total activity is recovered in the microsome fraction. On the basis of enzyme activity per unit weight of protein, this is the only fraction in which DPN-cytochrome c reductase is clearly concentrated; the concentration in the mitochondria does not appear to be significantly greater than in the whole homogenate. Hogeboom ('49) has reported a similar distribution pattern for rat liver fractions obtained from hypertonic sucrose, but his data suggest that the enzyme is concentrated not only in the microsomes but also, to a lesser degree, in the mitochondria.

Spectral observations on the cytochrome components of liver

Methods and materials. Both a direct vision prism spectroscope and a Beckman model DU spectrophotometer were employed for studies on the absorption spectra of the liver preparations. Examinations with the spectroscope were made on whole homogenates and on suspensions of the fractions in 0.05 M glycylglycine or phosphate buffer at pH 7.0. The oxidized spectra were observed in the presence of air and the reduced spectra in an atmosphere of nitrogen after addition of solid dithionite ($Na_2S_2O_4$).

To make the liver preparations sufficiently clear optically for spectrophotometric studies, solutions of sodium desoxycholate were used in a manner similar to that described by Ball, Strittmatter and Cooper ('51) for a heart muscle preparation. The homogenate or liver fraction was dispersed in ice-cold 0.05 M glycylglycine buffer, pH 7.0, containing sufficient sodium desoxycholate to give a final concentration of 1%. These somewhat turbid solutions were centrifuged at 110,000 times the force of gravity for 15 minutes at 0–5°C. After centrifugation each tube contained a very small sediment at the bottom, topped by a clear, colored supernatant and a very thin, white, scum-like layer at the surface. Relatively heavy tan sediments (about 5% of the total solids) were obtained from the whole homogenate and nuclear fractions; the minute sediments from the mitochondria and microsomes were, respectively, tan and almost colorless.

For spectrophotometric analysis, samples of the clear supernatants were carefully removed by pipette and placed in special vessels consisting of a Thunberg tube fused to the top of a standard Beckman corex cuvette (light path of 1 cm). When atmospheres other than air were required, the vessels were three times evacuated, flushed and filled with the desired gas. Substrates, if any, were then added from the hollow stopper and absorption measurements were made in the spectrophotometer at room temperature. Readings were made every 1 or 2 mμ in regions of maximal and minimal absorption and at 5 mμ intervals elsewhere. The slit width was 0.06 mm between 700 and 625 mμ, and 0.04 mm between 625 and 380 mμ. The wave length scale of the spectrophotometer was calibrated against various lines of a mercury lamp.

Absorption spectra of liver homogenates. No absorption bands were visible when the homogenates were examined in the presence of air with a hand spectroscope. When reduced with dithionite, the preparations showed a very feeble cytochrome a-cytochrome oxidase band at 605 mμ and a broad intense band at 549–565 mμ, centered about 555 mμ. Another diffuse feeble band was seen at 522–530 mμ; this band is usually ascribed to the β bands of the cytochromes.

When similarly examined with the hand spectroscope, a desoxycholate supernatant prepared from the homogenate showed the same absorption spectrum in the oxidized or reduced state as was given by the original homogenate under the same condition. This agreement between the absorption spectra of a liver preparation and the corresponding desoxycholate supernatant was observed also with the liver fractions to be described later.

The spectrophotometric findings confirm these observations. In figure 2 are plotted the absorption curves obtained with the desoxycholate supernatant from a homogenate, in the oxidized and reduced states. The concentration of the supernatant, expressed as grams of fresh liver represented by 1 ml of the solution, was 0.125. The oxidized curve (curve 1) shows no important peaks of absorption except a typical Soret peak at 412 mμ. The slight elevations at 575 and 535 mμ may be ascribed mainly to oxyhemoglobin derived from the few erythrocytes that contaminate the preparation. In the reduced state (curve 2) there are two broad peaks centered at 555 and 525 mμ, respectively, and the Soret peak is at 420–423 mμ.

The breadth of the first two peaks suggests that there are contributions from several absorbing substances. In view of the low hemoglobin concentration indicated by curve 1, reduced hemoglobin, which absorbs maximally at 555 and 530 mμ, can be responsible at most for only a small portion of these peaks. The main portion of the 555 mμ absorption therefore represents other, intrinsic hemochromogen material in the preparation.

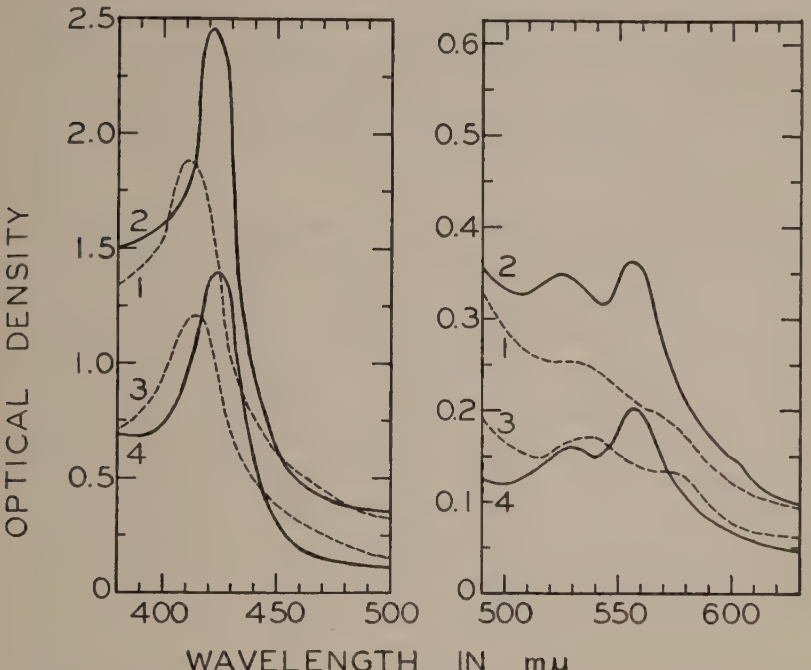

Fig. 2 Absorption spectra of rat liver homogenate and nuclear fraction. Curves 1 and 2 are spectrophotometric observations on a desoxycholate supernatant (concentration 0.125) prepared from rat liver homogenate (preparation H) as described in the text. Curves 3 and 4 are observations on a desoxycholate supernatant (concentration 0.25) prepared from the nuclear fraction (N). Curves 1 and 3 represent the oxidized state and curves 2 and 4 the reduced state after reduction by dithionite under nitrogen.

Absorption spectra of the nuclear fraction. When observed with a hand spectroscope after reduction with dithionite, suspensions of fraction N (nuclei plus cellular debris) showed absorption bands like those of the homogenate, except that no distinct 605 mμ band was visible. The oxidized and reduced absorption curves for a desoxycholate supernatant of fraction N (concentration of 0.25) are plotted in figure 2 as curves 3 and 4, respectively. This fraction contains all of the erythrocytes which contaminate the homogenate, hence the oxidized preparation possesses distinct oxyhemoglobin peaks at 576 and 540 mμ. The spectrum of the reduced preparation has absorption maxima at 555 and 530 mμ, but unlike the corresponding homogenate it shows no clear sign of a peak in the 605 mμ region.

Absorption spectra of the mitochondria. Examination of suspensions of mitochondria (fraction L) in the oxidized state with the hand spectroscope revealed no absorption bands, but the bands seen with reduced suspensions indicated the presence of a complete "normal" cytochrome system. The α bands of cytochrome a-cytochrome oxidase, cytochrome b and cytochrome c appeared at 605, 562 and 550 mμ, respectively, and the fused β bands of these components caused a diffuse feeble absorption in the 520–532 mμ region. There was no indication of any substance absorbing maximally between the α bands of cytochromes b and c.

Observations on desoxycholate supernatants of the mitochondria (concentration of 0.50) with the Beckman spectrophotometer are plotted in figure 3. Curve 1, obtained in the oxidized state, shows no definite elevation in the 575 mμ region, indicating that the preparation is fairly free of hemoglobin. The slight elevations at about 565 and 530 mμ are similar to those seen with cytochrome c in the oxidized state. When the preparation was reduced with dithionite in a nitrogen atmosphere (curve 2), there appeared a cytochrome a-cytochrome oxidase band at 605 mμ and a sharp cytochrome c peak at 551 mμ. The shoulder at 562 mμ may be ascribed to the presence of cytochrome b in a concentration considerably lower than that of cytochrome c. That cytochrome c is in preponderance is further evidenced by the fact that the peak representing the fused β bands is composed almost entirely of a maximum at 522 mμ, where cytochrome c absorbs, with only minor contributions from the other cytochromes.

It has been observed spectroscopically in succinate oxidase preparations from heart muscle (Ball, '38; Keilin and Hartree, '39) that succinate, like dithionite, readily reduces both cytochromes b and c, whereas such reagents as ascorbate and p-phenylenediamine which also reduce cytochrome c do not readily reduce cytochrome b. The effects of these reagents on the mitochondrial desoxycholate supernatant were tested to establish whether the substance absorbing at 562 mμ is indeed

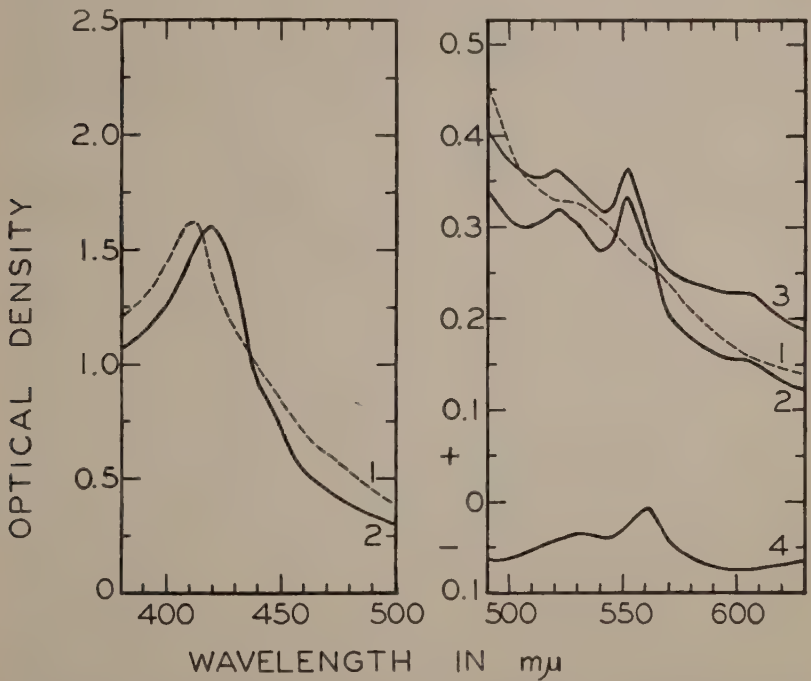

Fig. 3 Absorption spectra of rat liver mitochondria. These spectrophotometric observations were made on a desoxycholate supernatant (concentration 0.50) prepared from the rat liver mitochondrial fraction (L). Curve 1 represents the oxidized state and curve 2 was obtained after reduction under nitrogen with either dithionite or 0.013 M sodium succinate. Curve 3 was obtained with 0.013 M p-phenylenediamine as reducing agent. Curve 4 was obtained by subtracting the readings of curve 3 from those of curve 2.

cytochrome b. The data obtained are shown also in figure 3. The absorption curve obtained with 0.013 M sodium succinate as reducing agent was identical with curve 2, obtained with dithionite, i.e., both the cytochrome c peak and the suspected cytochrome b shoulder appeared. In curve 3, obtained with 0.013 M p-phenylenediamine as reducing agent, the cytochrome c peak is fully developed but no shoulder is seen on the curve. If 0.007 M freshly prepared ascorbic acid solution was used instead, the curve obtained was similar to curve 3. If the readings of curve 3 are subtracted from curve 2, a plot of the difference will be the absorption spectrum in the reduced state of the substance responsible for the 562 mμ absorption. This plot (curve 4) shows maxima at 561 and 530 mμ, which are the wavelengths of maximum absorption for cytochrome b.

Absorption spectra of the microsomes. Oxidized suspensions of the microsomes (fraction M) when viewed with the hand spectroscope showed no absorption bands in the visible part of the spectrum, but when reduced with dithionite there appeared a strong band centered at 557 mμ and a feeble band at 527 mμ. The band at 557 mμ differed from the band seen in this region in the original homogenate: it was distinctly narrower and appeared to be caused by a single absorbing substance rather than by a mixture of compounds.

The absorption spectra of a microsomal desoxycholate supernatant (concentration of 0.50) are shown in figure 4. Curve 1, representing the preparation in the oxidized state, has no band in the 575 mμ region; hence no appreciable amount of hemoglobin is present. A Soret band appears at 414 mμ and there are slight humps at 565 and 530 mμ. In curve 2, obtained when the preparation was reduced with dithionite under nitrogen, there is a sharp peak at 557 mμ and a lesser peak at 527 mμ, while the Soret peak is shifted to 424 mμ. These spectra are typical of a hemochromogen, and it therefore appears that the major pigment of liver microsomes is a hemochromogen resembling the ''normal'' mammalian cytochromes in its absorption spectrum but readily distinguishable from them. The properties and distribution of this pigment, which has tentatively been termed "cytochrome m," are described elsewhere (Strittmatter, '52; Strittmatter and Ball, '52). None of the "normal" cytochromes are spectrophotometrically discernible in the microsomes, but the presence of small amounts of cytochromes b and c would be masked by the relatively intense absorption of the microsome hemochromogen.

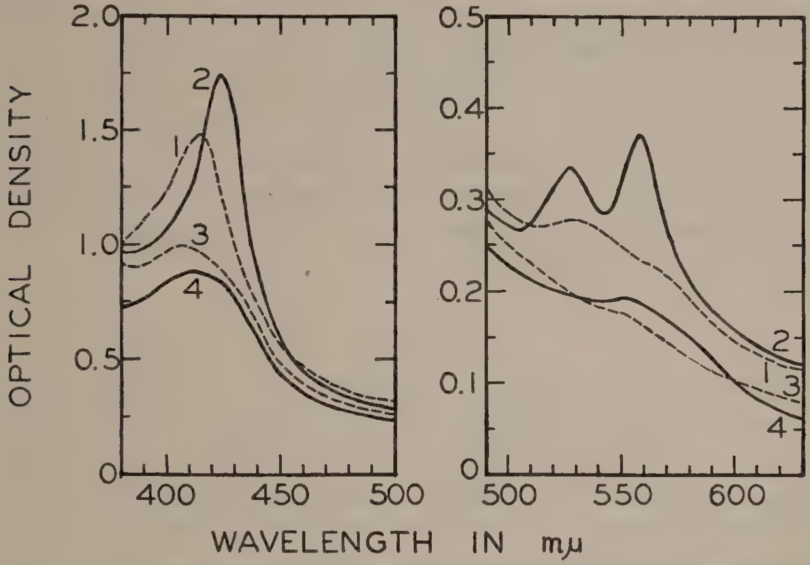

Fig. 4 Absorption spectra of rat liver microsomes and of the supernatant fraction. Curves 1 and 2 are observations on a desoxycholate supernatant (concentration 0.50) prepared from the rat liver microsomal fraction (M). Curves 3 and 4 are observations on a concentrated supernatant fraction (concentration 0.25) obtained in the liver homogenate fractionation. Curves 1 and 3 represent the oxidized state and curves 2 and 4 the reduced state after reduction by dithionite under nitrogen.

Absorption spectra of the supernatant fraction. The final supernatant of the liver fractionation (fraction S) showed no absorption bands in either the oxidized or reduced states when observed with the spectroscope, even when the fractionation procedure was altered to yield a more concentrated supernatant. The absorption curves obtained from a concentrated supernatant (concentration of 0.25) with the spectrophotometer are plotted in figure 4. In the oxidized spectrum, given by curve 3, there appears to be a Soret peak at 405 mμ, suggesting that the preparation contains heme compounds. Absence of a 575 mμ peak indicates that no appreciable amount of hemoglobin can be present. In curve 4, representing the reduced supernatant under nitrogen, the broad low hump near 550 mμ suggests that the pigment content of this fraction may include small amounts of various hemochromogen substances. The important point, however, is that this supernatant and hence also the original homogenate are essentially free of extracellular hemoglobin. The cytochrome-type spectra found in the mitochondria and microsomes therefore result from intrinsic pigments of these particles, and are not caused by adsorbed hemoglobin or compounds arising by denaturation or decomposition of hemoglobin.

DISCUSSION

It is clear from the results presented here that the unusual cytochrome spectrum displayed by whole liver is not to be interpreted as signifying that the liver possesses an oxidative pathway distinct from the usual cytochrome system. The findings reported here not only confirm and extend those of earlier investigators that the mitochondria are the chief site of oxidative processes in the liver but they also show that the mitochondria contain the familiar complement of cytochromes. The unusual cytochrome spectrum of liver is to be ascribed to the presence in the liver microsomes of a hemochromogen distinct from those found in the mitochondria. This pigment, which we have labeled cytochrome m, possesses in the reduced state a broad intense band centered at 557 mμ. It thus absorbs in the region between cytochromes b and c and causes the whole perfused liver to exhibit a general absorption throughout the region 550–565 mμ in contrast to the two-banded spectrum in this region shown by certain other tissues (cf. MacMunn, 1886).

The function of cytochrome m is unknown. Its concentration in liver is of catalytic proportions, being several times that of cytochrome c in this organ (Strittmatter, '52). In view of the marked ability of the microsomes to catalyze the interaction of reduced DPN with cytochrome c, one may well inquire whether cytochrome m plays any part in this catalysis. It has been demonstrated that cytochrome m can be reduced by DPN·H, though the reaction of its reduced form with cytochrome c has not been clearly proven. The work of Mahler, Vernon and Sarkar ('51) indicates that the cytochrome c reductase obtained by them from heart muscle is a flavoprotein. The nature of the cytochrome c reductase of liver microsomes has not been demonstrated and indeed the physiological function of this enzyme in the microsomes remains a puzzle. As is shown here, no cytochrome c can be detected spectroscopically in the microsomes; the mitochondria on the other hand are relatively rich in this component. Furthermore, as the data collected in table 6 demonstrate, the capacity of the microsomes to transfer electrons from reduced DPN to added cytochrome c is out of all proportion to their ability to pass these electrons on to oxygen. Two of the possible explanations for this seemingly anomalous situation may be

TABLE 6

Enzymatic activities in rat liver fractions

ENZYME SYSTEM	RELATIVE ACTIVITIES EXPRESSED IN TERMS OF MICROLITERS O_2 CONSUMED PER HOUR PER GRAM OF FRESH LIVER IN:	
	Mitochondria	Microsomes
Succinate oxidase		
(without added cytochrome)	4,810	280
(with added cytochrome c)	9,460	480
Succinate dehydrogenase	4,010	430
Cytochrome oxidase	18,100	1,400
DPN-cytochrome c reductase [1]	10,920	27,980

[1] Calculated for 37.5°C. from data obtained at 23°C. by assuming that the normal ΔQ_{10} of 2 holds for this enzymatic reaction, and that the transfer of four electrons is equivalent to the one molecule of O_2.

mentioned. First, the electrons removed from reduced DPN may somehow be transferred to the mitochondria and carried to oxygen through the comparatively rich cytochrome system in those particles. Second, the electrons available from reduced DPN may not be funneled through cytochrome c to oxygen but may instead be utilized for purposes of reductive synthesis. This second explanation seems more logical to us. If it is correct, then the microsomes may play a role in reductive reactions involved in the synthesis within the liver of such compounds as fatty acids and cholesterol. Cytochrome m forms an oxidation-reduction system with an E_0' at pH 7.0 of -0.12 v. (Strittmatter and Ball, '52). With this low oxidation-reduction potential, cytochrome m would be well suited to participate in reductive synthetic reactions.

Any consideration of the relationship of microsomes to mitochondria must take into account the significant differences between these two kinds of particles. The data presented here confirm previous findings that the microsomes possess more lipid, more cytochrome c reductase, and less oxidative activity than the mitochondria. In addition, it is shown here that the microsomes are rich in a hemochromogen, cytochrome m, which cannot be detected spectroscopically in the mitochondria. It has been suggested that the microsomes are disintegration products of mitochondria (cf. Green, '52). If this is the case, it would appear necessary to suppose that the mitochondrion is a heterogeneous particle and that the portions fragmenting to form microsomes differ markedly in composition and enzymatic activities from the remainder and from the parent mitochondrion. We would favor the interpretation of the data to suggest instead that the mitochondria and microsomes represent two separate types of cellular particulate matter.

SUMMARY

1. The cytochrome system of liver has been studied with the aid of rat liver homogenates and liver cell fractions prepared by differential centrifugation in isotonic sucrose media.

Spectroscopic and spectrophotometric examinations indicate that a complete "normal" cytochrome system is concentrated in the mitochondrial fraction. The anomalous cytochrome spectrum of liver is attributable to an intrinsic cytochrome-like component of the microsome fraction.

2. The composition of the liver fractions has been determined in terms of dry weight, protein and lipid content.

3. Studies on the intracellular distribution of oxidative enzymes are reported. Cytochrome oxidase, succinate dehydrogenase and succinate oxidase are associated almost exclusively with the mitochondria, while DPN-cytochrome c reductase appears to be concentrated only in the microsome fraction.

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THE OCCURRENCE OF β-GLUCOSIDASE IN DIGESTIVE JUICE OF THE COCKROACH, PERIPLANETA AMERICANA L.

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TWO FIGURES

INTRODUCTION

The belief is prevalent that β-glucosidase as a digestive enzyme is comparatively rare in occurrence in animals; to date it has been reported from the adult honey bee (utilization studies only, Vogel, '31), snail *(Helix)*, crayfish (see Prosser et al., '50, for a summary and references) and woodlice *(Porcellio* and *Armadillidium)* (Newcomer, '52). As far as the writer is aware, there is no experimental evidence to indicate the presence of a β-glucosidase in sea clams although Lavine ('46) states that the style contains "presumably a β-glucosidase active toward β-glucosidic linkages." The following report is concerned with the occurrence and partial characterization of a β-glucosidase in the roach, *Periplaneta americana* L.

METHOD

Crude extracts of the upper quarter to one-half of the ventriculus (midgut) including the 8 pyloric caeca of adult cockroaches, *Periplaneta americana* L., were prepared in the following manner. Fresh tissue was dissected from the animal without rupturing the walls of the caeca, dehydrated *in toto* in acetone, air dried, finely ground, suspended in distilled water and allowed to stand 12 hours before use to allow free distribution of the enzyme. Portions of the suspension were then incubated for various periods of time in buffered solutions with arbutin, cellobiose, or salicin as substrate (100 mg/10cm^3 total) under toluene at 30°C. and the formation of glucose determined by alkaline copper reduction (Folin, '29). Approximate final concentrations of the buffers were: acetate, 0.1 N; citrate, 0.1 N; phosphate, 0.066 M and phthalate, 0.2 M. It must be emphasized that only crude extracts were used throughout these experiments; no attempt at purification was made.

Experimental

Optimal pH range. To determine the optimal pH range over which this β-glucosidase hydrolyzes β-glucosides, a series of tests was made using equal amounts of enzyme at pH intervals of 0.4 units throughout a pH range of 2.8 or 3.0 to 6.8 or 7.2. In order to compare determinations from different series wherein the amount of enzyme was not the same due to the use of crude extracts, a relative basis of comparison was utilized. In any one series, that pH at which the greatest amount of hydrolysis was obtained was assigned a value of 100% conversion under the particular conditions of the experiment (time, buffer, substrate concentration, and enzyme concentration). The amount of hydrolysis at the other pH's within the same series was expressed as a percentage of the 100% value. Using a criterion of optimal pH as that pH exhibiting greatest hydrolysis plus all other pH's showing 75% of this level, the approximate optimal pH ranges for the various buffers are shown in figure 1: acetate, 4.8–6.6; citrate, 4.2–6.2; phosphate, 4.2–6.4; phthalate, 4.6–6.2. If the criterion of optimum is lowered to include 50% hydrolysis of the greatest amount (Michaelis and Davidsohn, '11), the optimal range is likewise extended in both directions. Due to the fact that each point in figure 1 represents an average of 4 or 5 single determinations of various series and that the 100% level of hydrolysis does not always occur at the same pH, none of the points actually reaches the top of the graph. In various series using acetate buffer the 100% level of hydrolysis fell at 4.8, 5.2, 5.6, and 6.0; using citrate, at 4.4, 4.8 and 5.2; phosphate, 4.8, 5.2 and 5.6;

phthalate, 4.8, 5.2 and 6.0. These ranges could possibly be used as a more limited definition of the optimal pH range.

There is uncertainty as to specifically where particular foods are digested in the digestive system of the roach (Abbott, '26). Wigglesworth ('27a) has made observations on the pH of the crop and midgut of the roach and found it to average 5.2 and 6.2, respectively, both of which fall within the optimal range of activity of this β-glucosidase as herein described.

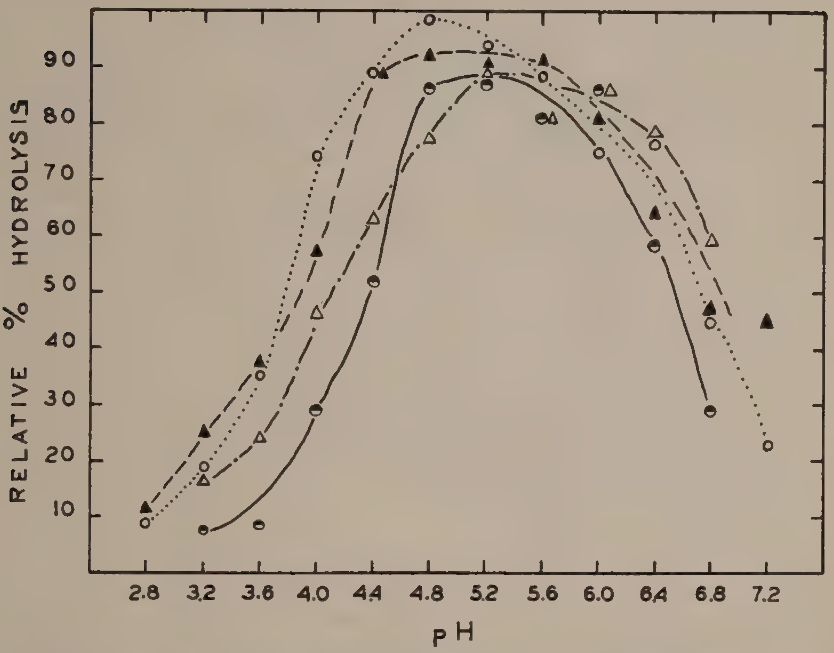

Fig. 1 Relationship between pH and degree of hydrolysis using various buffers: Acetate —.—.—; citrate —————; phosphate ......; phthalate ————. Substrate: 100 mg salicin/10 cm³ total. In any one series, that pH exhibiting the greatest degree of hydrolysis was arbitrarily designated 100% under prevailing conditions and all others in the same series (enzyme concentrations being equal) were expressed as relative percentages of the highest level. Each point represents an average of 4 or 5 determinations.

Velocity of hydrolysis. No attempt was made to determine definite values for the enzyme efficiency, salicin factor or salicin value of this enzyme due to the use of crude extracts only. Neither was it deemed desirable to create a new standard of velocity applicable only to these experiments. The relative relationship between enzyme concentration and degree of hydrolysis is shown in figure 2 wherein the concentration of enzyme was doubled respectively in order from D to A. From

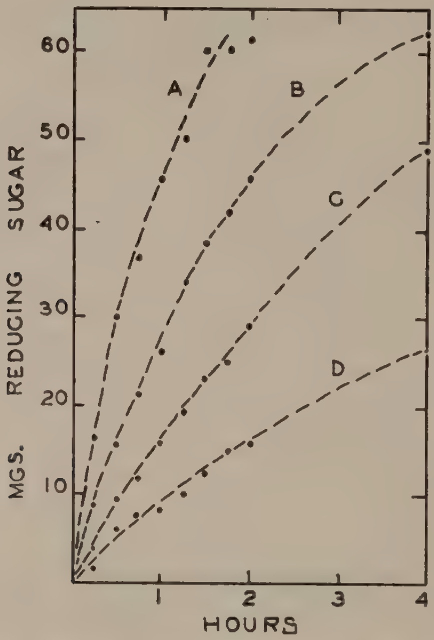

Fig. 2 Relationship between concentration of enzyme and velocity of hydrolysis. Substrate: 100 mg salicin/10 cm^3 total; buffer: phosphate at pH 5.2 Enzyme concentration progressively doubled from D to A (A having 8 × concentration of D). One hundred per cent hydrolysis of 100 mg salicin should theoretically yield 62.9 mg glucose.

the data upon which figure 2 is based, the average rate of conversion throughout the first 2 hours was as follows: A — 1.04 mg salicin/min.; B — 0.60 mg/min.; C — 0.36 mg/min.; and D — 0.21 mg/min.; the greatest rate occurred in the initial 15 minute period of A where it was 1.79 mg salicin/min.

Inhibition by heavy metals. Exposure of the enzyme to 0.1 N concentrations of $AgNO_3$, $CuCl_2$ and $HgCl_2$ for two hours prior to addition to substrate resulted in over 90% inhibition by silver and mercuric ions and 55–58% inhibition by cupric ions. See table 1. This inhibitory action is thought to be due to the formation of a salt (Veibel, in Sumner and Myrbäck, '50).

Acceleration by toluene. Veibel ('37–'38) has shown that saturation of emulsin solutions with toluene to prevent growth of microorganisms caused an increased activity of the enzyme upon certain substrates as measured polarimetrically. No such activation was demonstrated with this enzymatic preparation therefore toluene was used to inhibit activity of microorganisms during incubation in these studies.

TABLE 1

Inhibition by heavy metals

Results expressed as mg of reducing sugar. Substrate: 100 mg salicin/10 cm^3 total; buffer: phosphate, 5.0 pH. Equal concentration of enzyme

INCUBATION PERIOD AT 30° C.	0.1N $AgNO_3$	0.1N $CuCl_2$	0.1N $HgCl_2$	CONTROL
hours	*mg*	*mg*	*mg*	*mg*
2	1.0	6.0	0.2	13.5
4	1.5	9.3	1.0	22.5
8	2.0	13.5	1.5	32.0

TABLE 2

Comparison of substrates

All results expressed as mg of reducing sugar. Buffer: phosphate, 5.2 pH

INCUBATION PERIOD AT 30° C.	ARBUTIN 100 mg/10 cm^3	CELLOBIOSE 75 mg/10 cm^3	SALICIN 100 mg/10 cm^3
hours	*mg*	*mg*	*mg*
0	0	29 [1]	0
2	20.9	62.5	17.0
4	32.0	74.0	31.0
6	56.8	81.0	47.0

[1] This is slightly above the theoretical value of 26.7 mg for this particular concentration of cellobiose based on data from Bates et al. ('42).

Inhibition by formaldehyde. A 70–80% (Borkorny, '19) inhibition upon enzymatic activity was demonstrated by exposing the enzyme to 1% formaldehyde for two hours prior to adding it to substrate. Similar exposure of the enzyme to 0.1% formaldehyde produced no inhibition.

Variety of substrates. No striking difference in rate of enzymatic hydrolysis of arbutin, cellobiose or salicin is observed. See table 2. One disadvantage in using cellobiose as substrate in combination with alkaline copper reduction is that it reduces the copper reagent; salicin (the standard substrate) and arbutin do not.

Place of origin. To prove that this β-glucosidase is a component of the extracellular digestive juice, some experiments were run using the clear, brown fluid within the lumina of the caeca. Hydrolysis was obtained in all cases. For routine work it is easier to remove all the caeca, with the juice within, and prepare the extract as described above. The enzyme evidently is secreted by the caeca and poured into the midgut.

DISCUSSION

The exact physiological importance of β-glucosidase as a digestive enyme in making β-glucose available to the cockroach is at present unknown. Carbohydrases already described as occurring in various species of cockroaches are: amylase, maltase, invertase, lactase and lichenase (Plateau, 1876; Swingle, '25; Wigglesworth, '27b; and Schlottke, '37). The close association of this species with human habitation probably lessens but not necessarily eliminates the necessity of its feeding upon foods containing large amounts of natural β-glucosides even though they are widely distributed throughout plant materials. Of the three substrates used here, arbutin and salicin occur naturally in the bark and leaves of various trees and shrubs. On the other hand, in order for cellobiose to be made available for possible utilization by animals such as the roach, cellulose must first be acted upon by cellulase. An animal possessing β-glucosidase and not cellulase theoretically could (with other conditions of availability and activity being acknowledged) digest naturally-occurring β-glucosides such as arbutin and salicin. The possession of cellulase in addition to β-glucosidase theoretically could increase the range of utilizable β-glucose-forming substrates by the inclusion of cellulose which exhibits an extensive natural distribution. Since β-glucosidase occupies a similar position relative to cellulase as maltase does to amylase, it might be expected that β-glucosidase and cellulase would exhibit a close association in distribution among animals. This situation has been shown to be true to some extent (Prosser et al., '50). To date there has been no satisfactory demonstration of endogenous cellulase in the digestive juice of the cockroach. Roaches are geologically an old and successful group; their success undoubtedly depends to some extent upon the diverse nature of their food supply. In general, the greater the diversity of digestive enzymes possessed by a species, the greater the diversity of foods which can be utilized; this condition in turn could and probably does contribute materially to its biological success.

SUMMARY

1. The presence of a β-glucosidase in the digestive juice from the caeca of the cockroach, *Periplaneta americana* L., is demonstrated.

2. This enzyme hydrolyzes arbutin, cellobiose and salicin. With salicin as a substrate its optimal pH range is between approximately 4.2 and 6.6.

3. The action of this β-glucosidase is inhibited by 0.1 N concentrations of copper, mercury, and silver salts and by 1% formaldehyde. An acceleration by toluene could not be demonstrated.

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EFFECTS OF SHEATH REMOVAL ON THE SCIATIC OF THE TOAD, BUFO MARINUS

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ONE FIGURE

INTRODUCTION

In the light of the considerable evidence (e.g., Feng and Liu, '49, '50; Crescitelli, '51), still accumulating (Shanes and Berman, '53; Shanes, '54), for the large resistance to diffusion exerted by the peripheral sheath of connective tissue (i.e., the epineurium) surrounding the vertebrate nerve trunk, this structure should be removed if its properties are not to be confused with those of the fibers themselves in *in vitro* studies.

Our early work with desheathed nerves from the bullfrog, R. catesbiana, demonstrated an important limitation of this preparation which had been overlooked previously, viz., an instability, reflected by a large uptake of medium, which apparently is restricted to the interstitial space (Shanes, '51). This has recently been confirmed (Lorente de Nó, '52) and described in detail (Shanes, '53). Methods have since been found which will reduce or prevent such swelling (unpublished). Nevertheless, a preparation which would be stable without the use of special solutions appeared desirable. This has been found in the sciatic of the tropical toad, Bufo marinus. Some of its characteristics are the subject of this brief report.

METHODS

General. The unbranched, uniform segment available from the toad sciatic is shorter than that obtainable from large bullfrogs — frequently less than a centimeter long. Nevertheless, it sufficed for experiments on sodium exchange. For other measurements the entire length of the nerve distal to the junction of the spinal nerves, including the tibial and peroneal branches, was employed.

The sheath was removed as previously described for bullfrog nerve (Shanes, '53). This consisted of girdling the nerve proximally, cutting branches short, and drawing the sheath distally.

A rough estimate of sheath thickness was made by measuring the diameter of the nerve, lying in a pool of Ringer's, before and after desheathing, or by comparing the diameter of a desheathed nerve with its intact mate. The susceptibility of such measurements to many variables requires that they be regarded merely as indicating the order of magnitude.

Experimental temperatures were usually between 24 and 26°C. The Ringer's solution was the same as that employed for frog nerve (Shanes, '52).

Weights. The ends of the nerves were ligated with 3 mil. tantalum wire, which served for handling purposes. The sciatic and its attached wire were blotted on filter paper and quickly weighed on a 250 mg Roller-Smith torsion balance with a vernier of 0.1 mg/division. Reproducibility of weighings was within 1%. At the end of a series of weighings, the wire ligatures were weighed and corrected for.

Spike amplitude. Nerves, either blotted gently on filter paper or drained of excess Ringer's, were laid in air on parallel silver wires fixed in position on a lucite block. Supramaximal condenser discharges were applied to the proximal segment and the spikes were picked up on the tibial and peroneal branches at least 1 cm from the distal end. A Tektronix Type 122 differential preamplifier was used in conjunction with a DuMont 304H oscilloscope and 264-B voltage calibrator.

Potassium. Individual nerves were mounted on glass rods so as to hang completely free in a large volume of Ringer's. The latter was about 1000 times the volume of the immersed tissue. Oxygenation and stirring was accomplished by bubbling oxygen through the solution.

At the end of the soaking period, each nerve was shaken for 24 hours in 5 ml of distilled water, which completely removes the potassium. The extract was analyzed with a Beckman no. 10300 flame spectrophotometer as previously described (Shanes, '52).

Sodium exchange. This study was carried out with long-life Na^{22}. The original shipment from Oak Ridge was neutralized with NaOH and diluted a hundredfold in Ringer's. Water and other necessary constituents were added to assure a radioactive solution of exactly the same composition as the inactive Ringer's. From the estimate supplied with the radioactive shipment, as well as by actual measurement with gas flow counters previously found to have an over-all counting efficiency of 50%, the "hot" Ringer's contained approximately 1 μC/ml of Na^{22}.

Methods essentially the same as in our earlier study of intact bullfrog nerve (Shanes and Berman, '53) were employed in the current investigation. Individual sciatics were exposed for 20 hours to radioactive Ringer's, blotted, given a quick rinse in inactive Ringer's, and inserted in a specially designed unit (Shanes, '52). This unit permitted ca. 1 ml of inactive solution, agitated by bubbling O_2, to surround single nerves, to be removed for counting, and to be replaced by fresh solution. In this way, the time course of exchange could be followed on the same nerve by taking counts on the successive samples collected. The radioactivity remaining in the nerve at the end of the run was determined from the count of the 24 hour water extract; in this case Ringer's was added to the planchet to the same extent as present in the media samples to correct for possible absorption. From the media and nerve data so obtained, the radioactive sodium left in the nerve after increasing intervals in Ringer's, relative to the amount initially present, was reconstructed (fig. 1). We have already pointed out that neither the long preliminary soaking of nerves in "hot" solution, nor the small accumulation of activity in the bathing solution between successive collections, is sufficient to alter the kinetics of ion exchange (Shanes and Berman, '53).

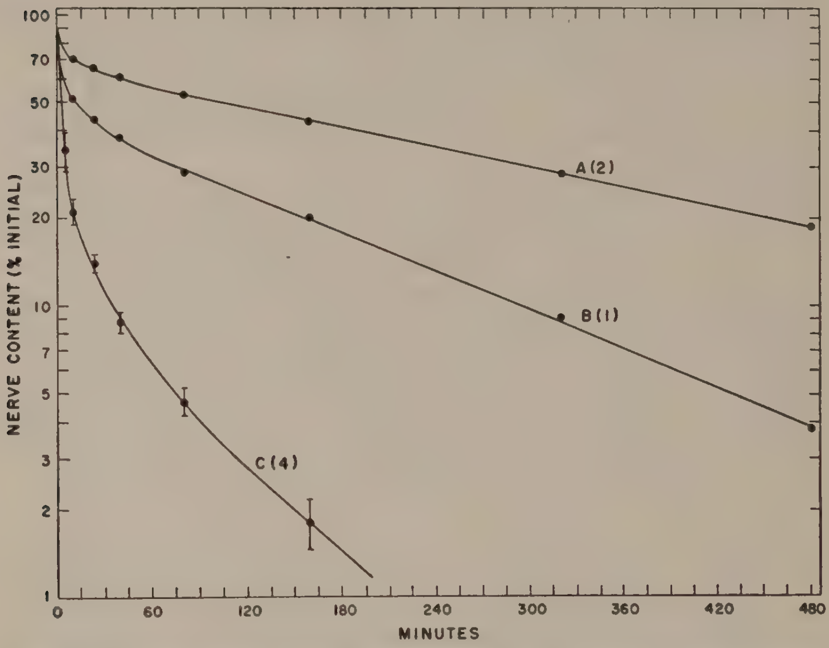

Fig. 1 Typical curves showing the decline in the radioactive sodium content, of intact (A and B) and desheathed (C) toad nerves, by exchange with the inactive sodium of the surrounding Ringer's solution. (A) Mean of two nerves with approximately the same sheath thickness, viz., 70 μ; total diameter averaged 1 mm. (B) Curve for a single nerve with a sheath thickness estimated at 135 μ; over-all diameter 920 μ. (C) Mean of 4 desheathed nerves with an average diameter of 750 μ; the vertical lines at each point represent the standard error.

RESULTS

Weights. In sharp contrast to the situation in bullfrog nerve, desheathed sciatics from B. marinus do not gain weight over the periods of time studied. As may be seen in table 1, no systematic change in weight is evident over as long as 44 hours. The same table illustrates that the spike amplitudes remain relatively constant for at least 19 hours. In the first two nerves, which were weighed repeatedly during 44 hours, no loss in spike amplitude was evident by the end of this long period; in fact, the same desheathed nerves, checked after a total of 96 hours in Ringer's at room temperature, gave spikes 73 and 88% of the amplitudes found at the time of sheath removal. Both sets of data therefore indicate that the desheathed sciatic from B. marinus is particularly stable and therefore more suitable for experimental study than that from R. catesbiana.

TABLE 1

Weight and spike amplitude of desheathed toad nerves as a function of duration of exposure to Ringer's solution measured from the time of epineurium removal

NERVE	WEIGHT					SPIKE		
HOURS:	0	1.5	19	28	44	0	19	44
	mg	*mg*	*mg*	*mg*	*mg*	*mv*	*mv*	*mv*
1.	23.4	23.3	24.9	22.9	21.2	14.7		16.9
2.	21.1	21.2	22.2	21.2	19.4	13.4		14.7
3.	25.7	25.7	24.4			17.7	24.6	
4.	29.5	30.1	29.0			20.0	16.6	
5.	28.2	27.7	32.0			26.2	31.5	
6.	29.9	29.4	28.8			28.8	29.2	
7.	26.4	26.5	25.9			15.8	18.8	
8.	23.3	23.4	24.0			20.4	21.5	
9.	21.3	21.2	21.7			21.6	21.6	
10.	24.6	24.5	23.9			25.6	26.2	

Spike amplitude. Table 2 illustrates the large increase in the measured spike amplitude when the sheath is removed. This is readily understandable as the consequence of the elimination of an appreciable shunt from around the fiber-containing core. In keeping with this view, spikes measured with unblotted sheaths, that is, with the excess fluid merely drained off, are lower than those obtained after blotting the nerves on filter paper. In other words, the sheaths of the former are a greater shunt than in the latter, a fact clearly reflected by the significantly larger ratio of the spike of the desheathed nerve to that of the intact preparation (see columns D/I and S/S′).

Table 2 also gives the percentage wet weight of sheaths, measured after being blotted on the outer surface, relative to the initial total blotted wet weight of nerve. It is seen to average almost 25% of the nerve mass, and hence must represent a substantial fraction of the extracellular space. As will be shown in the discussion, the estimate of the relative sheath volume, calculated from the spike amplitudes with and without sheath, agrees with that based on the weights.

TABLE 2

Spike amplitudes, immediately before and following removal of the epineurium from blotted or unblotted toad nerve, and the sheath weight relative to that of blotted intact nerve

	SPIKE (BLOTTED)			SPIKE (UNBLOTTED)			SHEATH WEIGHT
	Intact (S′)	Desheathed (S)	S/S′	Intact (I)	Desheathed (D)	D/I	
	mv	*mv*	%	*mv*	*mv*	%	%
	12.7	27.3	215	14.3	32.8	229	21.9
	16.4	21.8	133	8.6	21.9	255	27.0
	19.1	39.1	205	10.5	17.1	163	24.0
	10.9	22.3	205	8.6	21.0	244	24.0
	23.6	34.6	147	11.0	28.6	260	21.8
	19.1	28.2	148	9.5	28.6	301	25.3
	19.5	24.6	126	7.1	28.6	403	17.7
	13.2	28.2	214	8.1	33.4	412	24.6
	10.0	14.5	145	12.4	29.6	239	
	18.6	39.1	210	10.0	23.8	238	
	17.7	24.6	139	10.5	24.8	236	
	14.5	34.6	239				
	17.3	29.1	168				
	14.5	29.1	201				
Mean	16.2	28.4	175	10.1	26.4	266	23.3
S. E.			3.2			22.2	

Potassium. In table 3 are given data on the potassium contents, of appropriately paired intact and desheathed nerves, at the time of dissection and after 24 hours in Ringer's at

25°C. As usual, nerves taken from the same animal are compared on the same horizontal line. These data show that neither the intact nor the desheathed nerves lose potassium over the times studied. Comparison of the figures for intact nerve with those of the desheathed sciatic shows values almost 15% greater for the latter. Additional data are necessary to establish the significance of this difference; nevertheless, it is noteworthy as the possible consequence of the removal of a structure containing less potassium/gm wet weight than the fiber-containing core — a reasonable possibility in the light of the considerable non-cellular structure in the sheath. The percentage sheath weight (table 2), and the mean potassium levels with and without sheath, suggest that the epineurium contains half as much potassium as the desheathed nerve per unit weight.

TABLE 3

Potassium contents, per gram wet weight, of freshly dissected and 24 hour, Ringer's soaked intact and desheathed toad sciatics. Data for nerves from the same animal are on the same horizontal line

FRESH		24 HOURS	
Intact	Desheathed	Intact	Desheathed
µM/gm	*µM/gm*	*µM/gm*	*µM/gm*
35.1		38.6	
29.5		30.5	
33.3		33.6	
	42.3		43.3
	39.7		38.1
	31.9		29.9
		35.2	39.6
		33.4	42.3
		38.2	41.5
Mean[1]		34.2	38.7

[1] Of all data for intact and desheathed nerves.

Sodium exchange. The constancy of the weight of desheathed toad nerve is indicative of a fixed geometry. By virtue of this, the role of the sheath as a diffusion barrier may be evaluated, with considerably more certainty than in bullfrog nerve, by comparing ionic exchange with and without the sheath. In the desheathed frog sciatic, the enlargement of the extracellular space leaves the possibility that the increased diffusion rates in this preparation are due to an increase in the effective diffusion area resulting from larger interfibrillar spaces (Shanes, '53; Shanes and Berman, '53).

In figure 1 the sodium exchange curves of intact toad nerves are compared with the mean of desheathed mates. The similar data for two intact nerves of approximately the same sheath thickness (ca. 70 μ) are averaged together as curve A. A single nerve, with a sheath estimated as 135 μ thick, gave curve B. The results for 4 desheathed nerves were practically identical and therefore averaged together as curve C. The rate of sodium exchange is clearly much greater after removal of the sheath.

The exchange curves for intact toad nerve are essentially the same as for bullfrog nerve in being divisible into an initially fast, smaller component and a secondary major, slow phase. The linearity of the latter on semilogarithmic coordinates demonstrates a simple exponential decline which usually was not quite attained by R. catesbiana sciatics (Shanes and Berman, '53). In the examples given in the figure, the thicker sheath is associated with a larger initial component, in keeping with the earlier conclusion that this relatively fast emission occurs from within the sheath itself (Shanes and Berman, '53). Calculation of the sheath volume relative to that of the total for the intact nerve from the dimensions gives values which agree well with those obtained by extrapolating the linear portion of curves A and B to zero time.

The emergence of Na^{22} from the desheathed toad nerves is not a simple exponential. However, data collected at more frequent intervals at early times are required to more clearly define the shape of the emission curve of this preparation.

DISCUSSION

It is remarkable that nerves from genera so closely related as Rana and Bufo differ so strikingly in their lability following removal of the epineurium. Until considerable comparative data are available, the characteristics which have been found cannot be attributed to habitat, nor can they be considered peculiar to the species, genus, or family. Consequently, unless the stability of desheathed nerves from still other species of frogs and toads has actually been examined, earlier observations on the sciatics from the two genera, or even from different species within the same genus, cannot necessarily be considered comparable to the current results.

In discussing the weight changes and appearance of their desheathed preparations, which were from Asiatic frogs and toads, Feng and Liu ('49) did not state specifically whether these particular observations may have been restricted to one species. Their general remark that most of the work was on toad nerve, as well as the similarity of their description of the appearance of desheathed nerve to our findings with the desheathed B. marinus sciatic, make it probable that their discussion was based on toad nerve. If such is the case, their statement that desheathed nerve undergoes a 10% increase in weight indicates that the sciatics from another species of toad are less stable following sheath removal.

A rough calculation can be made to determine the sheath volume required to account for the smaller spikes in intact nerve for comparison with the weight data given in table 2. Thus, the spike, S, as measured with external electrodes, is smaller than that, A, measurable across the membrane, by the ratio of the external resistance, R_e, to the sum of axoplasmic resistance, R_i, and R_e (Hodgkin, '47):

$$S = \frac{R_e}{R_e + R_i} \cdot A = \frac{A}{1 + R_i/R_e} \qquad (1)$$

Equation (1) will serve equally well for intact and desheathed nerve except, of course, S is larger for the latter since R_e is larger. Designating by primes the values for the intact nerve, we have

$$R_i/R_e = 1 - (S'/S)\ (1 + R_i/R_e') \qquad (2)$$

Taking R_i/R_e' to be the same as in intact bullfrog nerve, viz., 0.33 (Lorente de Nó, '47), we obtain, from the spike data of blotted nerves (table 2), $S'/S = 0.57$, whence $R_i/R_e = 0.24$ and $R_e/R_e' = 1.37$. Thus, it may be stated that the external resistance of intact nerve is about 25% less than that of the desheathed nerve. If the sheath is regarded as a simple parallel resistance shunting the fiber-containing core, its conductance is found to be approximately 25% of the total. Hence, if the longitudinal specific resistance is about that of the fiber-containing core, the cross-sectional area (or the weight, when the density of the sheath is about that of the core) is also about about 25%. This figure is in good agreement with the mean percentage weight of the sheath obtained from the data in table 2. Consequently the conclusion appears justified that the increase in spike height upon removal of the sheath is due largely, if not completely, to the elimination of its substantial shunting action.

The agreement of the calculated and measured volumes of the sheath is also a justification of the assumption that the longitudinal specific resistance of this structure is comparable to that of the fiber-containing core. The radial specific resistance must be much greater by a factor given by the diffusion coefficient in water to that in the sheath, which is of the order of several hundredfold (Shanes and Berman, '53). Such a difference in longitudinal and radial resistivities is suggestive of extremely flat structures, arranged coaxially, as the source of the high diffusion resistance of the sheath. Hodgkin ('51) has called attention to descriptions of flattened "endothelial" cells in the perineurium.

By the process of curve fitting for a homogeneous cylinder described previously (Shanes and Berman, '53), the emission curves for intact nerve are found to indicate that the slow emergence of the radioactive sodium is equivalent to an exchange (i.e., self-diffusion) coefficient, in the fiber-containing core, of $0.6\text{–}0.8 \times 10^{-6}$ cm^2 min^{-1}. This is three- to fourfold smaller than for bullfrog nerve, or a thousandfold smaller than in free aqueous solution.

It is now quite evident that the exchange coefficient of the core, derived by the curve fitting procedure referred to above, is virtual rather than real. That is, it is not an actual characteristic of the fiber-containing core. If it were, the desheathed nerves would be expected to lose sodium at a rate comparable to that of the slow component of the intact trunk. Such obviously is not the case in figure 1. Preliminary calculations give a coefficient of 4×10^{-5} cm^2 min^{-1} for the core, i.e., 50 times greater than for the intact trunk but still 20 times less than in free solution.

The objection could be raised that some undetected damage or increase in fiber spacing might have occurred during sheath removal to elevate the penetrability of the desheathed nerve. However, it is shown in the following communication that bullfrog epineuria alone, filled with radioactive solution, have the diffusion characteristics of the intact nerve. This makes a similar situation all the more likely for the toad sheath. It therefore appears that the available data suffice to establish the epineurium as the cause of both the fast and slow components of the emission curves in intact nerve.

The difference in the stability of desheathed toad and frog nerves, to which attention has been directed in this research, presents an interesting problem in comparative anatomy and physiology. Preliminary histological sections [1] indicate a thin, residual peripheral layer of connective tissue [2] surrounding the desheathed toad nerve which is absent in the desheathed bullfrog trunk. Whether this is the cause of the stability, or merely reflects a difference in the cohesiveness or other property of toad connective tissue which actually is responsible for the constancy in weight, cannot be answered at present.

The author is indebted to Mr. M. Berman for assistance with these experiments.

[1] The author is greatly indebted to Dr. James B. Longley, of the Laboratory of Pathology and Pharmacology, for making these available.

[2] This may be the "perineurium" distinguished by Key and Retzius (1876) from the epineurium in mammalian nerve.

SUMMARY

Desheathed toad sciatic nerve is a stable preparation compared to similar preparations from the bullfrog. The weight, spike amplitude and potassium content remain constant for many hours. Removal of the epineurium nearly doubles the spike height, as measured with external electrodes and with the nerves mounted in air; this is accounted for by the shunting action computed from the relatively large volume of the sheath. The emergence of Na^{22} by exchange with Na^{23} occurs in the intact toad nerve in two stages, as in the bullfrog sciatic. The major exchange occurs with a fiftyfold smaller exchange coefficient than in the desheathed preparation, and is one-thousandth as great as in free solution.

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SODIUM EXCHANGE THROUGH THE EPINEURIUM OF THE BULLFROG SCIATIC

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ONE FIGURE

INTRODUCTION

The entry and exit of ions and sucrose in bullfrog nerve have been shown to occur in two stages, a small but relatively rapid one, attributed to the appreciable volume of the sheath, and a second, larger but slower, which involves the interstitial space (Shanes and Berman, '53). A possible explanation of the slow component of penetration appeared to be extremely small spacing between the nerve fibers which could reduce greatly the area available for radial transfer. On this basis, the faster rates in desheathed nerve (Feng and Liu, '49 and '50; Crescitelli, '51) were attributable to the interstitial expansion indicated by a weight increase (Feng and Liu, '49; Shanes, '53).

The finding that a desheathed preparation which does not gain weight still has a substantially greater penetrability (Shanes, '54) more clearly implicated the sheath. However, the possibility remained that spacing, or another factor, had been altered during epineurium removal so as to enhance rates, without being reflected in a subsequent change of weight. This alternative could be eliminated by a demonstration that the sheaths alone can duplicate the kinetics seen with intact nerve. This has now been accomplished, and is the subject of this brief report.

METHODS

All experiments were carried out at 25°C. with individual peripheral sheaths removed from the unbranched segment of sciatics taken from Rana catesbiana. They were employed either inside or right side out, one of each type being prepared from the same animal.

The inverted sheath was obtained with the least amount of manipulation. It was produced by the usual technique (Feng and Liu, '49; Shanes, '53) of girdling in a proximal area and pulling it over itself as it was taken off the distal segment; the tibial and peroneal branches were removed first to minimize tension on the sheath.

An additional operation was necessary to restore the normal orientation of the epineurium. The peroneal and tibial branches were left intact and the sheath drawn over them until their origin was exposed; the desheathed segment was then cut away and the branches, now covered with two layers of sheath, were pulled distally along the surface of filter paper, as described by Crescitelli ('51), which thus supplied the necessary traction to turn the epineurium right side out again. Preliminary attempts at reversal of the sheath by pulling with forceps gave the leakiest preparations.

The epineuria were loaded with radioactive sodium directly and indirectly. In both cases, a 1 or .5 ml tuberculin syringe, with a blunted no. 24 needle in view under a dissecting microscope, was used. The proximal end of the sheath was tied to the needle with 3 mil tantalum wire and the usual inactive or 1 μc/ml of active Ringer's (Shanes, '54) was carefully introduced into the lumen. The tantalum wire also served to close the free end, which had been left with a small plug of the fiber-containing core which now was removed, and to tie off the region slightly beyond the needle's tip. The bags so formed were 1 to 1.5 cm long and averaged 1.2 mm in diameter. They were mounted individually on glass rods in the usual way and placed in radioactive Ringer's.

The sheaths filled with Ringer's were left in "hot" solution for 18 hours, as were the intact nerves. Sheaths to be used within several hours after removal from freshly dissected nerves were filled directly with radioactive Ringer's and left in the same active Ringer's for another hour. The customary series of consecutive collections was made to follow the emergence of Na^{22} in inactive Ringer's, and the previously described counting and other methods were employed to reconstruct the curve of ion emission (Shanes and Berman, '53; Shanes, '54).

RESULTS

The data are summarized by the curves given in figure 1. It is immediately apparent that the exchange curves for the sheaths (A, C, D) closely resemble that for intact nerve (B). All show a smaller relatively fast initial component followed by a larger slow one. The first component appears to be little different whether the sheaths are right side or inside out. The second component is a simple exponential, whereas for the nerves there is a definite although small deviation from linearity on the semi-logarithmic plot, as found with chloride (Shanes and Berman, '53). Experiments with desheathed bullfrog nerve, to be described elsewhere, provide a basis for the slight non-linearity; sheathed toad nerve gives a strictly exponential time course (Shanes, '54). However, the difference between the emission curves of the sheaths and intact nerves is exceedingly small and may be neglected for present purposes. What cannot be overlooked is the general duplication of the kinetics of ion exchange in the whole nerve by the sheath itself.

The duplication obtained is remarkable from the standpoint of the possibilities of damage to the sheath during the course of removal. That damage or deterioration did occur is indicated (1) by the consistently faster exchanges obtained with sheaths subjected to the additional operation required to restore them to their original orientation (cf. curves A and C in fig. 1) and (2) by the faster loss of activity from 18 hour preparations as compared with fresh ones (cf. curves A and D in fig. 1). Sheaths subjected to both less favorable treatments lost activity most rapidly; the poorest of these still retained over 10% of the original activity at the end of an hour in frequently replaced inactive Ringer's. Nevertheless, the majority of the data for the sheaths lie within the variability of the emission curves for whole nerve.

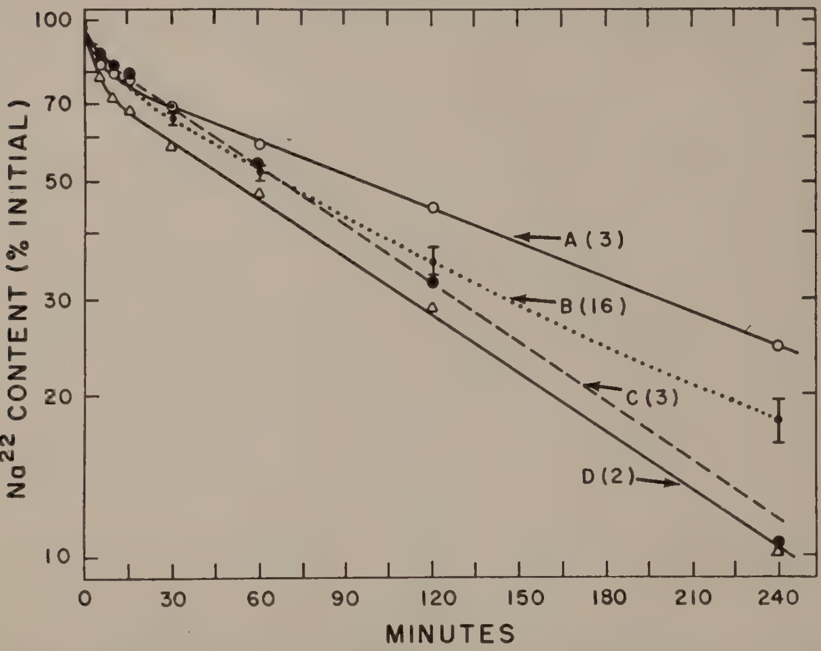

Fig. 1 Relative decline in the content of Na^{22}, by exchange with Na^{23} in the surrounding Ringer's, of (A) inverted sheaths, 2 hours after removal from the animal, (B) intact nerves, after 17 hours' preliminary equilibration in "hot" Ringer's, (C) right side out sheaths, 2 hours after removal from the animal, and (D) inverted sheaths, after equilibration as for (B). Parenthetical figures give the number of series contributing to each mean curve.

DISCUSSION

The direct analytical data in this and earlier papers are necessary and sufficient to attribute to the epineurium the major responsibility for both the initial rapid and subsequent slow emergence of diffusible substances from intact nerve. In the light of the direct experimental evidence now available, the indirect studies presented by Lorente de Nó ('47, '50, '52) in support of the view that the epineurium is a negligible diffusion barrier must be rejected as invalid. Moreover, ample justification is now at hand for the view that the barrier action of the epineurium accentuates potassium concentration changes in the extracellular space resulting from potassium shifts in the fibers (e.g., during anoxia, post-anoxic recovery and drug action; Shanes, '48, '51, '52). The accumulating quantitative data will ultimately permit a more critical analysis of the relation between such potassium concentration changes and the accompanying bioelectrical phenomena.

The two-stage emission which has been obtained is that to be expected from a bag in which the major resistance resides in a peripheral boundary of finite volume. If the barrier were of negligible thickness, then the radioactivity would decline exponentially throughout with a time constant inversely related to the permeability (Shanes, '51, '52). However, the measurable volume of the sheath (Shanes and Berman, '53; Shanes, '54), because of its fivefold greater surface to volume ratio compared to the lumen as well as of its proximity to the external medium, undergoes the rapid loss of a large part of its activity seen as the initial component in the emission curve.

A previous rough estimate of the diffusion coefficient of the sheath, based on the time of disappearance of its component, indicated a value of the order of 1/500 of that for aqueous solutions (Shanes and Berman, '53). A better determination is now possible from the slow component of the emission curve, which can be shown to be described by

$$\ln(C/C_i) = -KAt/Vx = -2Kt/rx.$$

A is the surface area, *V* the volume and *r* the radius of the lumen; *K* is the exchange (self-diffusion) coefficient and *x* the thickness of the sheath; and C/C_i is the ratio of the activity in the bag at time t to that at zero time. The linear slope of the curves in figure 1, or the time constant, therefore give K. The time constants of curves A, C and D are 184, 125 and 120 minutes, respectively. Taking r as 0.6 mm and x as 40 μ (Lorente de Nó, '50), we obtain 6×10^{-7} to 10^{-6} cm^2/min for K, or about the same as the previous rough estimate and of the order of 1000 times less than for free self diffusion.

The source of such a low coefficient poses an interesting problem. The exchange and diffusion coefficients of Na, Cl and sucrose, relative to each other, are about the same as in aqueous solution (Shanes and Berman, '53). This renders unlikely the involvement of different numbers of available pores of molecular or ionic dimensions, or of passage through selective cell membranes in the sheath. One possibility is that a few pores of large diameter (relative to molecular sizes), as suggested by Feng and Liu ('49) from their observations of protein penetrability, represent a greatly restricted effective area for penetration. If these occur at substantial tangential distances from each other in the adjacent concentric lamellae, described by Key and Retzius (1876) as composing the sheath, an additional factor would be a greatly lengthened effective mean path. That the endothelial-like cellular elements of the sheath represent the major barrier is suggested by the observation that a substance which penetrates cells readily, CO_2, likewise enters the nerve trunk at rates approaching that in free aqueous diffusion (Fenn, '28). Failure to recognize this important distinction between the penetrability of "polar" and "non-polar" substances led Lorente de Nó ('50) to argue that CO_2 penetration indicates the pattern of entry for all substances. However, the later work of Fenn et al. ('34) has already demonstrated the considerably slower exit of ions from the intact nerve trunk.

The author is indebted to Mr. M. Berman for assistance with these experiments.

SUMMARY

The kinetics of the emergence of Na^{22} by ionic exchange from the bullfrog epineurium is practically identical with that for the intact nerve. This is considered conclusive proof that the sheath is the limiting factor in the entry and exit of experimental substances in the intact sciatic *in vitro*.

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EFFECT OF THYROID HORMONE ON MITOSIS AND REGENERATION IN CORNEAL EPITHELIUM[1]

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TWO FIGURES

Although the rôle of the thyroid in growth and differentiation of the individual is well established and its effect on the growth of some isolated tissues has been reported, its influence on growth of various parts of the body (Hammett, '27), and on metabolism (Smelser, '44) of even the same tissue in different parts of the body is not necessarily the same. Therefore, in a study of the significance of a number of factors involved in epithelial maintenance and repair of injuries, the importance of thyroid hormone in these processes in the cornea was included.

The corneal epithelium offers particular advantages in such studies, as the number of mitotic figures in a known area and the rate of mitoses is easily determined. It is an avascular structure so that variation in the rate of blood flow or number of capillaries in underlying tissue does not influence the rate of healing. Epithelial injuries heal rapidly and without contracture, due to the rigidity of the cornea, so that rates of epithelization, as well as of mitosis, can be readily studied.

EXPERIMENTAL

Male rats of the Sherman strain maintained on a stock diet, supplemented with fats, vitamins and minerals, were used

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[1] This investigation was supported by a grant from the Ely Lilly Company.

in this study. In all cases where comparisons were to be made between several groups of experimental and control animals, the litters were so divided that representatives were assigned to each experimental group.

Effect of hypo- and hyper-thyroidism on the healing of superficial corneal burns

Forty rats from 9 litters were used. Ten of these were thyroidectomized, at 7 days of age; 15 others received 0.2 mg thyroxin per 100 gm body weight, by injection, daily for 14 to 16 days prior to autopsy. When the animals were 5–6 weeks of age and the normal controls averaged 95 gm in body weight, thermal burns were made, under ether anesthesia, on the right cornea of each rat. The burns were inflicted by a 5 second application to the cornea of a Shahan thermophore, heated to 71°C. The applicator tip was curved to fit the rat corneas and burned a horizontal strip 1.5 mm wide across its full width. In this procedure the underlying stroma was not apparently injured. Such burns were completely covered by the regenerating epithelium in 15 hours. In these experiments the animals were autopsied 12 hours after the burn was made, when regeneration was nearly complete, the eye enucleated and fixed in Bouin's fluid, and the unhealed portion of the burn was drawn with the aid of a camera lucida. The area of the drawing was measured with a planimeter. If healing was delayed, a larger area than normal was found at this time — if it was hastened, a smaller than normal area was found or epithelization may have been completed. After the drawings were made, the corneas were embedded in paraffin and sections made at right angles to the burned strip. A series of sections taken at regular intervals across the width of the cornea were stained, so that the number of mitotic figures could be counted. The technical procedures used were the same as those described in greater detail earlier (Smelser and Ozanics, '44). A second series of experiments (24 animals) was conducted in exactly the same manner, except that the degree of healing was assessed by measuring the uncovered burn area, as seen in sections, rather than by the planimetry of drawings of the burn area.

Although body growth of the thyroidectomized rats was far below normal, averaging 46 gm at autopsy compared with 95 gm of their normal litter mates, the healing of the corneal burns was slightly more rapid than normal. The completeness of thyroidectomy was checked at autopsy, and only animals entirely devoid of thyroid tissue were included in the data. In hyper-thyroid rats however, the rate of regeneration of the burns was slower than normal, although considerable variation was found. The degree of hyper-thyroidism induced was marked, for these young growing rats, usually rather resistant to thyroxin, weighed on an average 10 gm less than their litter mate controls; body fat depots were markedly depleted, and a tremor was evident in most cases. The burn areas at the 12 hour stage were: thyroidectomized 399 mm^2; normal 453 mm^2; and hyperthyroid 501 mm^2. These figures represent the areas of camera lucida drawings of the burns, made at 30 × magnification. The results obtained in the second series of experiments, in which the residual burn area was measured differently, were in complete agreement with the above data.

Mitosis is not a prerequisite to the process of epithelization (Smelser and Pfeiffer, '48), and in some cases decreases during the period of cell movement (Arey and Covode, '43; Friedenwald and Buschke, '44). However, during the first 15 hours following thermal burns, a significant increase in the number of mitotic figures has been found in regenerating epithelium, in comparison with the other uninjured eye (Smelser and Ozanics, '44). Counts were made of the number of mitotic figures in the intact left, and burned, but nearly healed, right cornea of the normal, hypo-, and hyper-thyroid animals in these experiments. It will be seen (fig. 1) that more mitoses were found in the burned, than in the intact, corneas of the normal animals. In 62% of the cases this difference was statistically significant ($P < .01$). This elevation in the number of mitoses was somewhat less in the hyper-thyroid group, being found in only 43% of the cases. In two instances, the number of mitotic figures was less in the burned eye than in the normal. The expected rise in the number of mitoses in the corneas of the thyroidectomized animals was even more marked than in the normal, for over 87% of the cases showed this elevation. It appears that mitotic activity in the regenerating epithelium is not seriously interfered with by either thyroid excess or deficiency, although a tendency is apparent for mitotic activity, like epithelial movement, to be less in the hyper-thyroid animals.

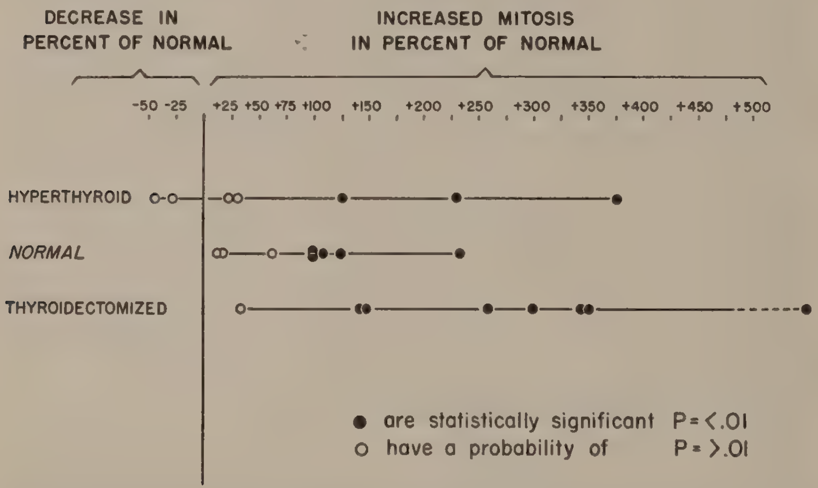

Fig. 1 The effect of hyper- and hypo-thyroidism on the number of mitotic figures in regenerating corneal epithelium is shown. The number of mitoses in a unit area of the corneal epithelium, 12 hours after a burn was inflicted, is expressed as the per cent of the number found in an equal area of the intact control cornea. In each case the right eye was injured and the left was intact.

Mitotic activity in the intact epithelium

Cell division in the intact corneal epithelium was studied in 76 animals. Six groups were prepared, and each litter was divided so that a member was represented in each group. The experimental groups were as follows:

(1) Normal
(2) Normal, plus colchicine
(3) Hypo-thyroid
(4) Hypo-thyroid, plus colchicine
(5) Hyper-thyroid
(6) Hyper-thyroid, plus colchicine

The colchicine, 0.3 mg per 100 gm body weight, was injected intraperitoneally 7 hours prior to autopsy. Hypo-thyroidism and hyper-thyroidism were induced as described above. At autopsy the eyes were enucleated, fixed in Bouin's fluid, and corneal strips approximately 1 mm in width were removed and prepared as whole mounts (Buschke, Friedenwald and Fleischmann, '43). Mitoses were counted in a band, outlined by an Ehrlich stop in the ocular, running the complete width of the cornea. The mitotic figures in several such bands were counted, and, in addition, the mitoses were classified according to stages (prophase, metaphase, anaphase and telophase) in many of the cases.

The data obtained are shown in figure 2, A and B. No evidence of a difference in the number of mitoses in the cornea of normal, hypo- or hyper-thyroid cases was found. Measurements were made of the corneal area in which the counts were made, so that a correction could be applied for variation in the size of the cornea which might affect the counts. No such correction was found necessary, for the corneal diameter of the markedly dwarfed thyroidectomized rats was only 4% less than normal. Obviously such counts as these are only of the number of cells actually in the process of mitosis at the moment of fixation. Variation in the rate in which cells entered the process of mitosis or the time consumed in cell division would not necessarily be revealed by such counts. For this reason the number of mitotic figures in the cornea of normal, hypo-, and hyper-thyroid rats treated with colchicine was counted.

It is assumed that colchicine, in this dosage, arrests the mitotic division in metaphase, and therefore that counts would represent the number of cells which entered the process of division during the period in which the colchicine was effective. To demonstrate that the colchicine was actually effective in all groups, the mitotic figures were classified according to their stages of division. Ratios were found of the number in early mitosis (pro- and metaphase) to those in late stages (ana- and telophase). In animals not treated with colchicine about one-half as many dividing cells were found in the late stages as in the early. The ratio of early/late mitotic stages were: hypo-thyroid 100/55, normal 100/53 and, a little higher, 100/67 in the hyper-thyroid cases.

The action of colchicine alters these ratios markedly by inhibiting the completion of mitosis, so that ratios of 100/9.4

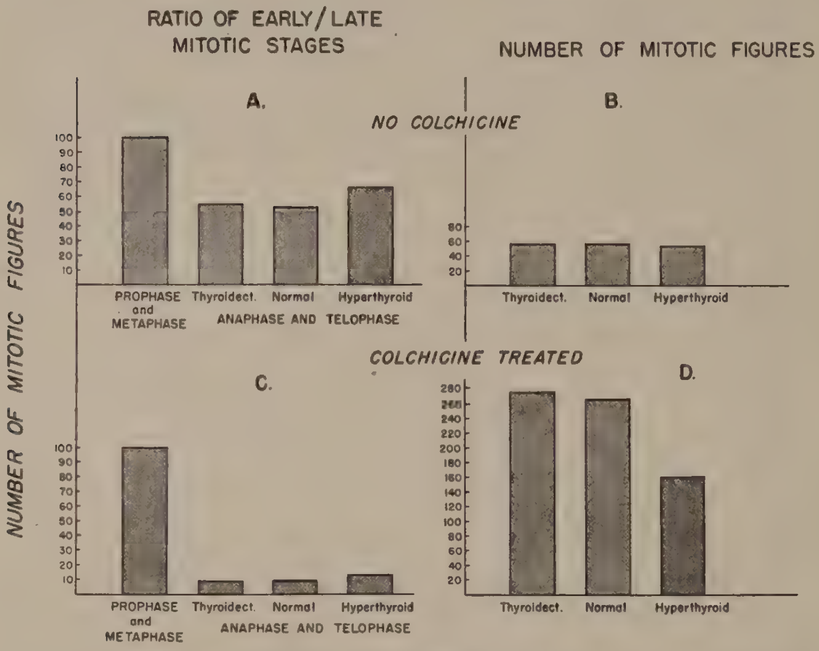

Fig. 2 The ratio of early/late mitotic stages found in the intact corneal epithelium of thyroidectomized, normal and hyper-thyroid rats is shown (A) and compared with similar data obtained in colchicine treated rats (C).

The number of mitotic figures found in a unit area of intact corneal epithelium of thyroidectomized, normal and hyper-thyroid rats is shown (B) and compared with similar data obtained in colchicine treated animals (D).

and 100/9.7 were obtained in the hypo-thyroid and normal cases. This ratio was again a little higher in the hyper-thyroid series where the number of early to late stages was 100/13.6, figure 2, C. If the rate of the mitoses is slow in hyperthyroidism, the larger number of late mitotic stages can be understood. Mitotic figures found in late stages of the colchicine-treated animals may represent the few which escaped this particular dosage of colchicine, or some which had passed the metaphase but failed to complete their division after the colchicine was given.

The number of mitotic figures found in the corneas of the three experimental, colchicine treated groups was not equal as they were when no colchicine had been given (fig. 2, B). The normal and hypo-thyroid corneas contained nearly an identical number of mitoses, 265 and 274 respectively per unit of the corneal area, whereas the number of mitotic figures in the hyper-thyroid group was 160 per unit area, figure 2, D. The probability that the difference between the normal or hypo-thyroid animals and the hyper-thyroid is significant, is $P = 0.02 - 0.05$. In all three groups, the colchicine treatment resulted in significantly ($P < .01$) higher mitotic counts. The low number of mitoses in the hyper-thyroid group is not therefore due to ineffectiveness of the colchicine for, in addition to the above evidence of its effectiveness, the toxicity of colchicine in hyper-thyroid animals was tested and found to be the same or greater than normal. Also the ratio of early to late mitotic stages and the structure of the metaphase was characteristic of cholchicine action. One is led to the conclusion therefore that excessive thyroid hormone decreases both the rate of entry of corneal epithelial cells into mitosis and the time consumed in the process.

DISCUSSION

Since the importance of thyroid hormone in growth and differentiation is accepted, it is but natural that it has been assumed that this stimulant of metabolic activity might also hasten the processes involved in healing and regeneration. Although Hammett ('26) suggested that thyroid hormone was more involved in growth (cell size as well as body size) than with cell division, the thyroid hormone has been suggested as an agent of value in stimulating tissue repair following injury (Arey, '36; Trowell and Willmer, '39). The data presented here do not lend support to such an idea. Indeed, it appears that healing of small areas denuded of their epithelium, as in these experiments, can proceed normally in the absence of the thyroid hormone, under which condition normal body growth is impossible. Possibly the thyroid hormone is of far greater importance to the active growth of connective tissues, such as bone and cartilage, than of epithelial tissues which may, in a more passive sense, simply keep pace with the growth of the substrate which they cover. Most of the references to the favorable effect of thyroid hormone on wound healing are concerned with growth of connective tissues. The epithelization of corneal burns of the hyper-thyroid group was apparently slower than of the normal and thyroidectomized animals although, due to individual variation, the delay was not statistically significant. This indicated delay may have resulted from the generally poor condition of the animals, as evidenced by depot fat depletion caused by administration of excessive amounts of thyroxin, although it does not seem likely that hyper-thyroidism limited cell movement by reducing available metabolites required in this process. The results on cell division in the regenerating epithelium also indicate that hyperthyroid animals are less able to react with the usual increase in the number of mitoses, following burns, than are thyroidectomized animals.

The effect of thyroid hormone on mitosis in the intact corneal epithelium appeared to be quite as definite as it was unlooked for. For although thyroidectomy markedly inhibited body growth, and undoubtedly fewer mitoses occurred in the epiphyseal cartilages and other structures than in the control animals, cell division appeared to be perfectly normal in the corneal epithelium. The eye as a whole, its neural elements and their connective tissue capsule grow quite normally in animals dwarfed by thyroidectomy (Hammett, '27).

The older work on the effect of thyroid hormone on mitosis is reviewed by Hammett ('26). In some recent experiments (Smith, '51), an increase in the number of dividing cells in the mouse ear epithelium was found following thyroid feeding. It was not determined whether this increase resulted from an elevated frequency of cell division or a slowing of the mitotic process. In addition, mitosis in a location such as the ear, may very well be more subject to changes in temperature capillary circulation or action of the autonomic nervous system than the cornea.

The use of colchicine in experiments such as these has been discussed by Buschke, Friedenwald and Fleischmann ('43). They point out that colchicine used in appropriate amounts blocks mitosis at the metaphase, without affecting the rate of entry of cells into the mitotic process. If excessive quantities of colchicine are administered, some decrease in the rate of entry into mitosis may occur. In the present experiments mitosis was apparently not blocked 100%, therefore it is felt that the amount of colchicine administered was not excessive. In addition, the dosage was considerably below those producing obvious toxic symptoms or death. If hyper-thyroid animals are relatively more sensitive to colchicine than normally, it is conceivable that the amount given may have affected the rate of entry of cells into mitosis, whereas it did not do so in the normal and thyroidectomized groups. However, our data, on the completeness of the mitotic block, do not show this to be so, figure 2, C. Furthermore, the rather limited tests of the toxicity of colchicine which were performed, revealed no great difference between normal and hyper-thyroid rats.

A preliminary account of these experiments was reported earlier (Smelser, '47), following which a note appeared (Fleischmann and Breckler, '47) in which similar studies were carried out on rats rendered hypo-thyroid with thiouracil, and, in 6 cases, by thyroidectomy. In these experiments a decrease in the number of colchicine arrested mitotic figures in the cornea of hypo-thyroid rats was found. No explanation of this discrepancy has been offered. It is therefore still more surprising to find in the present experiments that hyperthyroidism affects cell division in the same manner as was reported by Fleischmann and Breckler ('47) to occur in their hypo-thyroid rats. Very few differences between their experiments and these are obvious. In our studies the animals were much younger when thyroidectomized and were probably hypo-thyroid for a longer period. The present data are based on a much larger series of thyroidectomized rats, but no studies were made on rats rendered hypo-thyroid with thiouracil. The demonstration that the colchicine was equally effective in thyroidectomized and normal rats is important, in such experiments, for if, as occurs occasionally due to unknown causes, the colchicine does not block mitosis, low counts are obtained which would markedly reduce the average response. The studies of Fleischmann and Breckler ('47) on epithelial movement during healing were in agreement with those of our own reported here and in the preliminary communication. Unfortunately, no studies on the effect of hyper-thyroid states were reported by them.

CONCLUSIONS

1. Excess thyroid hormone tends to inhibit the processes of repair, both cell division and epithelization, following superficial corneal burns.
2. Thyroid deficiency, sufficient to markedly stunt skeletal growth, does not affect mitotic activity in the intact corneal epithelium, or epithelization and cell division following superficial corneal burns.
3. Excess thyroid hormone decreases the frequency of cell division and slows the mitotic process in intact corneal epithelium.

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